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## RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

Including

BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY,  
INTERNAL MEDICINE, PHYSIOLOGY, PSYCHIATRY,  
SURGERY, AND VETERINARY MEDICINE.

Volume II.

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ANNUAL PROGRESS REPORT  
1 July 1976 - 30 September 1977



## VOLUME II

(11) Sep 77 /

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(Projects and work units are  
listed in Table of Contents)

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VOLUME II

WALTER REED ARMY INSTITUTE OF RESEARCH  
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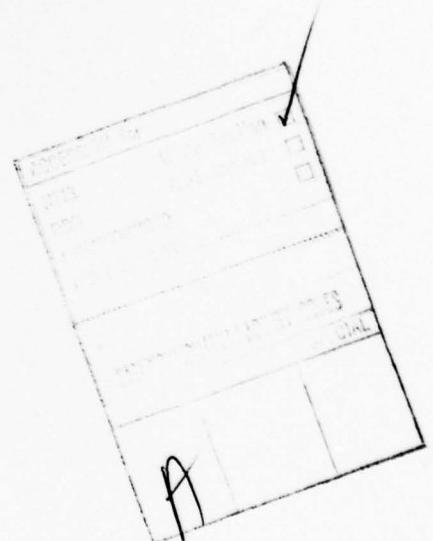
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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

The FY 7T report is included in this Annual Progress Report

SUMMARY

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report, and names of investigators are given at the beginning of each report.



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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup> DA OB 6489	2. DATE OF SUMMARY <sup>b</sup> 77 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMMARY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCFTY <sup>c</sup> U	6. WORK SECURITY <sup>c</sup> U	7. REGADING <sup>d</sup> NA	8. DISPN INSTRN <sup>e</sup> NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: <sup>f</sup> a. PRIMARY 62770A	PROGRAM ELEMENT PROJECT NUMBER 3M762770A802			TASK AREA NUMBER 00	11. LEVEL OF SUM WORK UNIT NUMBER 001	
b. CONTRIBUTING						
c. CONTRACTOR <sup>f</sup> CARDS 114F						
12. TITLE (Pecede with Security Classification Code) (U) Epidemiologic Studies of Military Diseases						
13. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>g</sup> 003500 Clinical Medicine 005900 Environmental Biology						
14. START DATE 72 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House			
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		
a. DATES/EFFECTIVE: NA	EXPIRATION:	FISCAL YEAR	PRECEDING 77	7	20. FUNDS (In thousands) 19	
b. NUMBER: <sup>h</sup>		CURRENT	78	9	30	
c. TYPE:	d. AMOUNT:					
e. KIND OF AWARD:	f. CUM. AMT.					
20. RESPONSIBLE DOD ORGANIZATION		21. PERFORMING ORGANIZATION				
NAME: Walter Reed Army Institute of Research ADDRESS: Washington, D.C. 20012		NAME: Walter Reed Army Institute of Research Division of Preventive Medicine ADDRESS: Washington, DC 20012				
RESPONSIBLE INDIVIDUAL NAME: Raptmund, Garrison, COL, MC 202-576-3551 TELEPHONE:		PRINCIPAL INVESTIGATOR (Purish SEAN II U.S. Academic Institution) NAME: Dobbs, Olin C., COL, MC TELEPHONE: 202-576-3553 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED] ASSOCIATE INVESTIGATORS NAME: Hodder, Richard A., LTC, MC NAME: Gaydos, Joel C., LTC, MC				
22. KEYWORDS (Pecede EACH with Security Classification Code) (U) Epidemiology; (U) Infectious Disease; (U) Data Bases; (U) Human Volunteer						
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Purish individual paragraphs identified by number. Pecede text of each with Security Classification Code.) 23. (U) To identify, define, and study known and potential causes of disability in military populations using relevant, existing epidemiologic techniques and developing appropriate new methodology. To apply this information to the prevention and control of disability in military populations. 24. (U) Contemporary epidemiologic methods are applied to causes of disability in military populations. Multidisciplinary collaborative approaches are utilized and new methods developed as required. 25. (U) 76 10-77 09 Analyses of data have been completed for: investigation of the swine influenza A outbreak at Fort Dix; an adenovirus Type 21 vaccine trial (immunogenicity and safety); an investigation of a hepatitis outbreak at Fort Richardson; surveillance for the development of hepatitis of an alert force deployed to Alaska; and, an investigation of a hepatitis outbreak at Tripler AMC. Analyses of data from the following infectious disease studies are in progress: determination of the prevalence of antibodies to adenoviruses in new recruits; determination of the prevalence of antibodies to polioviruses in new recruits; and, an adenovirus Type 21 vaccine trial (immunogenicity, safety, and efficacy). (The preceding studies are complementary to work described under DA OB 6513, Work Unit 135, entitled "Mechanisms of Transmission of Hepatitis Viruses," and DA OA 6441, Work Unit 130, entitled "Viral Infections of Man.") Analyses of data from the following studies using existing data bases are in progress: the determination of congenital malformation rates by time for several posts; description of renal disease admissions for Army active duty personnel; description of the Army's experience with Guillain-Barre syndrome; determination of the rate of occurrence of testicular tumors in an Army command; and, the identification and retrieval of data needed to study the development of malignancy in persons exposed to nuclear test shots. For technical report see WRAIR Annual Progress Report, 1 Jul 76-30 Sep 77.						

DD FORM 1498  
1 MAR 68

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

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Project 3M762770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Task 00 Military Preventive Medicine

Work Unit 001 Epidemiologic studies of military diseases

Investigators:

Principal: COL Olin C. Dobbs, MC

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1. Hepatitis A Outbreak, Fort Richardson, Alaska, August 1976 - April 1977

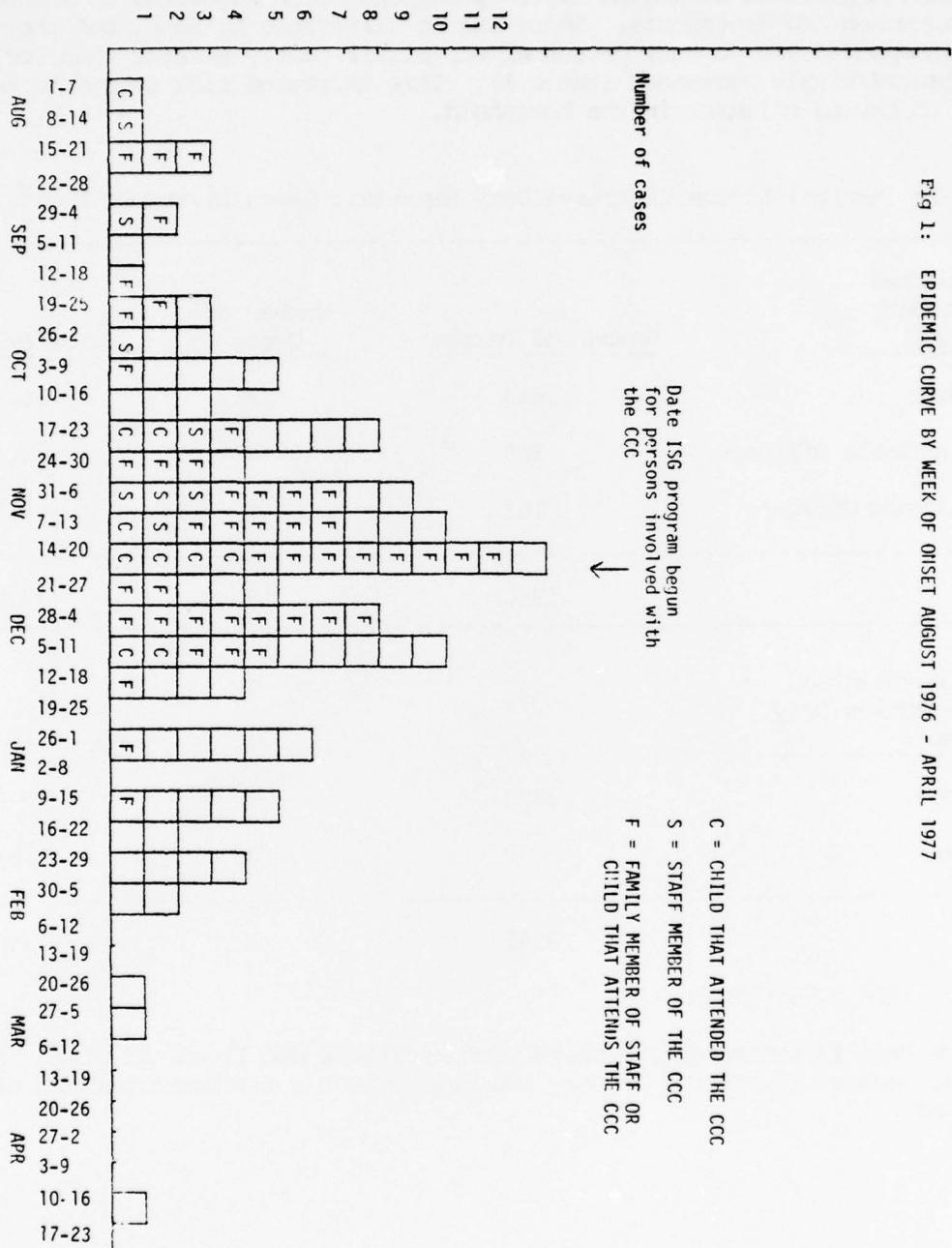
An increase in the number of cases of hepatitis among military personnel and their dependents at Fort Richardson, Alaska was investigated by the local Health and Environment Activity in the fall of 1976. The continued development of cases and the inability to determine a common source prompted a request for EPICON assistance. An EPICON team made visits to Fort Richardson in November and again in December. The team was accompanied by members of the Department of Virus Diseases during the December trip.

One-hundred-sixteen cases were identified from 1 August 1976 to 1 May 1977 in military active duty members and their dependents stationed at Fort Richardson and in civilians working on the post. The majority of the cases occurred during the months of November and December, with a median date of onset of 15 November (fig. 1).

Sera were collected from 61 of the cases contacted by the EPICON team. None were positive for HB<sub>s</sub> antigen, but seven (11%) were positive for HB<sub>s</sub> antibody. This suggested that the disease was due to hepatitis A virus. Eleven arbitrarily selected persons were tested for hepatitis A antibody (anti-HAV); ten (91%) demonstrated positive sero-conversion or sustained high titers of anti-HAV by the immune adherence method, indicating recent infection with hepatitis A virus. These findings confirmed the causative agent of the outbreak to be hepatitis A virus.

Fortunately the clinical expression of the disease was relatively mild, and there were no deaths. Only a small number of the cases were hospitalized, and these were either unaccompanied servicemen living in the barracks or pregnant dependents hospitalized as a precautionary measure by their obstetricians.

Fig 1: EPIDEMIC CURVE BY WEEK OF ONSET AUGUST 1976 - APRIL 1977



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The 116 cases, when characterized by age, sex, military status and rank, and then compared to estimates of the post population suggested an increased risk in women and dependents. There was no difference by rank, but the risk for accompanied active duty personnel was significantly greater than for unaccompanied/single personnel (table 1). This increased risk seemed to be related to having children in the household.

TABLE 1: Marital Status Of Active Duty Hepatitis Cases Living On Post<sup>#</sup>

<u>Accompanied Active Duty Personnel</u>	<u>Number of Persons</u>	<u>Number of Cases</u>	<u>Rate/1000</u>
Enlisted	1414	22	15.56
Company Grade Officers	269	4	14.87
Field Grade Officers	103	1	9.71
<b>TOTAL</b>	<b>1786</b>	<b>27</b>	<b>15.12*</b>

Unaccompanied and  
Single Active Duty  
Personnel

Enlisted	3873	8	2.06
Officers	73	0	---
<b>TOTAL</b>	<b>3946</b>	<b>8</b>	<b>2.03*</b>

\*  $\chi^2_C = 32.6$  p less than .001

<sup>#</sup> There were 12 active duty members with hepatitis who lived off post. Six of these became ill after 1 January and may reflect a different pattern of exposure.

Since there was a suspected association with children, a busy child care center (CCC) located on Fort Richardson was further investigated. Of the 116 total cases, eight were children who attended the CCC on a regular basis; two were children who used the CCC infrequently; eight were staff members; one case was the sibling of an attendee; and 44 were adults living in households where one or more children attended the CCC. A review of the medical records of the children in the households of the 44 adults, revealed several children with physician visits for flu-like illness compatible with mild hepatitis which could have gone unrecognized. Thus 63 (54%) could be directly or indirectly linked to the CCC (fig. 1).

Risks related to attendance at the CCC were determined by data abstracted from time cards kept on each child visit at the CCC. Time cards were examined on an alternate day sampling frame from September to November encompassing 35 surveyed days. The following information was obtained from the data on the time cards:

(1) Risk related to time of exposure in the CCC. Rates of hepatitis in children attending the CCC, according to hours of CCC exposure increased from 5.9/1000 for children in the CCC 1 to 20 hours to over 30/1000 for children there more than 60 total hours.

(2) Household risk by time of attendance and age of the attendee in the CCC. Table 2 shows the percentage of households with a case of hepatitis by 20 hour periods of total household attendance for households with children under three years of age and those with children over three years. There is a marked increase in risk with increasing hours of exposure in those households with younger children.

(3) Risk of hepatitis in households with an attendee at the CCC. All households in a representative section of the housing area were surveyed to determine if a household member attended the CCC and data collected were correlated with reported cases of clinical hepatitis. Households with a member attending the CCC were significantly more likely to have a case of hepatitis ( $p < .001$ ).

Further evidence of transmission in the CCC was provided by a serological survey of the staff of the Fort Richardson CCC, and the staff of the Elmendorf CCC (located on an air base directly adjacent to Fort Richardson). Two blood samples were obtained one in November and the second six weeks later. Twenty-one of the 30 persons tested at the Fort Richardson CCC were positive for anti-HAV (70%), while only 15 of the 38 tested at the Elmendorf CCC were positive (39%). These data suggest that transmission had occurred at the Fort Richardson CCC and that it had essentially terminated by the time of the first bleeding since there were no conversions during the 6 weeks between bleedings.

Control efforts consisted of dissemination of information to the community concerning hepatitis and its transmission. ISG administration to contacts was initiated early in the outbreak. In November ISG was made available for persons associated with the CCC. This included the staff, children attending the facility, and family members of the attendees. There is some evidence that the ISG

Table 2. Percentage of Households With At Least One Case of Hepatitis Based On Total Household Hours Of Exposure In The CCC. Comparison Of Those Households With A Child 1 Year Of Age Or By Attendance With Those Households With Only Children 3 Years And Over In Attendance

TOTAL CCC HOURS FOR THE HOUSEHOLD	HOUSEHOLDS WITH A CHILD LESS THAN 3 YEARS OF AGE ATTENDING THE CCC		HOUSEHOLDS WITH ONLY CHILDREN 3 YEARS OR OLDER IN ATTENDANCE	
	Total Number	No. With Case Hepatitis*	Total Number	No. With Case Hepatitis*
1-20	214	11	5.1	168
21-40	56	1	1.8	34
41-60	30	3	10.0	21
61-80	18	3	16.7	10
81+	47	11	23.4	45
TOTAL	365	39	10.7	278
				4
				1.4

\*Does not include Hepatitis in the household member attending the CCC.

program was helpful (fig. 1). In only eight households was there what appeared to be a secondary case, suggesting that in the household setting the administration of ISG to contacts was helpful.

The implications of this investigation are important. Child care facilities should be considered as different from schools when evaluating their potential for disease transmission and dissemination to the community. Young, pre-toilet trained children have a considerable capacity to transmit hepatitis virus. Additionally, since asymptomatic infections predominate in this young age group, many young children who are unknowingly sources of infection continue to have close contact with other young children and family members. The possibility of community spread underlies the importance of a case occurring in a child care facility. It is imperative that both the child care center staff and the medical community be aware of this potential.

## 2. Hepatitis at Tripler Army Medical Center, June 1977

The unusual occurrence of several cases of hepatitis B antigen negative hepatitis among the staff at the Tripler Army Medical Center (TAMC) in early June 1977 was investigated by the Health and Environment Activity and the hospital Infection Control Committee. A common source was not identified and a request for EPICON assistance was forwarded through channels. An EPICON team arrived at TAMC, Honolulu, Hawaii on 24 June 1977.

Seven cases of hepatitis with onset between 18 May and 7 June were recognized among the staff at TAMC. There had been no reported cases among the staff during the previous two years. There was no increase in the number of reported cases in Hawaii, either from the civilian or military communities during 1977.

HbsAg and Hbs antibody testing at WRAIR produced negative results on all six of the patients tested, suggesting that the outbreak was due to hepatitis A disease. Testing for antibody to hepatitis A has not yet been done.

Detailed interviewing of identified cases and other staff members failed to find other cases that were previously unrecognized and also failed to incriminate a common factor related to the cases. Possibilities of a food or water borne outbreak were examined with negative results. Person-to-person spread among the cases was also considered but was unlikely because of the similar dates of onset.

The seven cases among the staff occurred in two groups. Two of the cases were officers and the other five were enlisted corpsmen. The officers were both married nurses and lived off-post. The corpsmen, (four male, one female) were all single, lived in the barracks and were in Medical Company B. It is postulated that these two groups were exposed to hepatitis in different ways.

The enlisted personnel were most likely exposed in the barracks. Assuming that exposure in the hospital should be equal for ward personnel living in the barracks and out of the barracks, it is unlikely that all five of the cases would live in the barracks (see Table 1).

TABLE 1: Hepatitis In Medical Company B

	Hepatitis	No Hepatitis	Total
Lived In Barracks	5	107	112
Lived Off-Post	0	269	269
	5	376	381

p = .002 Fishers exact test

Table 2 shows a similar situation when only the enlisted personnel working on the involved wards are considered.

TABLE 2: Hepatitis In The 76 Enlisted Personnel Working On The Involved Wards By Residence In The Barracks

	Hepatitis	No Hepatitis	Total
Lived In Barracks	5	19	24
Lived Off-Post	0	52	52
	5	71	76

p = .002 Fishers exact test

Examination of the plumbing plans (renovated within the last two years), plumbing work orders, and water testing reports from the barracks revealed no evidence of contamination. There were no eating facilities in the barracks except for kitchens that were infrequently used.

There was considerable social interchange among the five patients who lived in the barracks. Three of the patients lived on the same floor in rooms next to each other (two were roommates at the time of the investigation, but had not been during the period of probable exposure). All four of the male enlisted patients were friends and shared drinks and "smokes". The female patient in the barracks was the girl friend of one of the male patients. All five of these

patients had their onset at approximately the same time suggesting that they all may have been exposed to a sixth person known to all of them. The previous roommate of one of the cases was discharged from the service for disciplinary reasons. He had close contact with the other five cases, but as far as they knew he was asymptomatic at the time he left the Army and Hawaii, on 7 June. There were apparently no other enlisted persons in this social group.

The two officers had no connection with each other, and only minimal contact with the enlisted personnel that were ill. One of the enlisted patients had worked on each of the wards that the officers worked on, but had little contact with the officers.

It is possible that some of the cases were exposed to patients with hepatitis on their respective wards. It is unlikely that all of the cases could have been exposed this way since infectious patients would have had to have been hospitalized on several wards at approximately the same time. There were no recognized cases of hepatitis on the involved wards except for ward B-18 which regularly houses hepatitis patients.

In summary, seven non-hepatitis B cases of hepatitis were recognized among staff working at TAMC. Ruling out exposure by a common source (food, water) in the hospital was felt justified by the small number of cases that occurred. The only viable hypothesis remaining supposes exposure on the wards to hospitalized patients with undiagnosed hepatitis (this is most likely for the nurses), and common exposure to an asymptomatic case in the barracks for the corpsmen.

### 3. Medical Surveillance Of An Alert Forces Group Deployed To An Area Hyperendemic For Hepatitis

The planned deployment of U. S. Army Forces Command and U. S. Readiness Command troops to Alaska in January 1977 for a month long "Jack Frost" exercise caused concern about the acquisition of viral hepatitis by these troops. Not only would most of these deployed soldiers spend time in staging areas in the vicinity of Anchorage, but many were going to isolated areas with poor sanitation and would be associating closely with Alaskan Scouts and local National Guard units, in areas where hepatitis is hyperendemic.

In order to determine if viral hepatitis is a potential threat during such exercises, the Surgeon, JFK Special Warfare Center, Ft Bragg, N. C. initiated a medical surveillance program. The objectives of the program were to determine the immunologic status of the troops participating in the exercise and to identify clinical illness that occurred during and shortly after the exercise. Two-hundred-seventy-five participants from Fort Bragg (264 men, 11 women) were studied.

Pre-deployment sera were obtained from 169 of the 275 individuals (61.4%) and post-deployment sera were obtained from 265 participants (96.4%). Either a pre- or post-deployment specimen was obtained from 273 of the 275 participants. Paired sera were available for 161 individuals.

Of the soldiers providing paired sera, none who were initially HBsAg negative became HBsAg positive, and none who were negative for antibody to HBsAg prior to deployment were found to have antibody on return to Fort Bragg. Therefore, no serologic evidence indicating acquisition of hepatitis B during the Jack Frost exercise was found. One individual was positive for HBsAg on testing both pre- and post-deployment sera, one other who provided only a post-deployment specimen was also positive (2 positive, 0.7%).

Of the 273 individuals from whom blood specimens were obtained, 42 (15.4%) demonstrated antibody to hepatitis B. There is an increase in the proportion of positive individuals by rank, both for the enlisted personnel and the officers. This is most likely a reflection of age and the likelihood of a previous tour overseas. The mean years of active duty was approximately twice as long for the group with positive results (11.7 years compared to 5.8 years for enlisted personnel, and 18 years compared to 9.3 years for officers).

Review of 225 medical records representing 82% of the group and 179 questionnaires (66%) revealed no illness suggestive of hepatitis. Questionnaires revealed mild upper respiratory infections to be the most common illness. Two individuals were hospitalized during the exercise, one for bacterial pharyngitis and the other for cervical strain.

In summary, the 15.4 percent hepatitis B antibody prevalence and the 0.7 percent HBsAg prevalence obtained in this study are comparable to percentages obtained in civilian and military populations in the U. S. No data is yet available on hepatitis A antibody. No clinically recognized hepatitis developed in this group.

#### 4. Incidence Of Testicular Tumor In Active Army Personnel

In January, 1977, the Epidemiology Consultant Service (EPICON), Division of Preventive Medicine, WRAIR, undertook an investigation of the incidence of testicular tumors in the U. S. Army at the request of the Office of the Surgeon General, Department of the Army. A retrospective study technically designed to identify all cases of this disease that occurred in active duty Army personnel from 1 Jan 72 through 31 Dec 76 was essentially completed by October 77.

The major data sources utilized to locate cases of testicular tumor diagnosed from 1972-76 were the Biometrics Division, Fort Sam Houston, Texas (IPDS), and the tumor registries at the U. S. Army teaching hospitals. Additional sources included the Naval Medical Data Services Center; Biometrics Division, Air Force Directorate of Health; DOD Central Medical Registry; and the AFIP G-U Tumor Registry. The first three of this latter group were contacted in an

attempt to identify Army personnel who were treated in non-Army medical facilities. The use of these diverse information sources has permitted the location of what is felt to be nearly 100% of Army personnel who developed testicular tumors during the five-year period 1972-76. Clinical information necessary to document each case was supplied largely by the Patient Administration Divisions and Tumor Registries at the various U. S. Army teaching hospitals.

The U. S. Army Military Personnel Center (MILPERCEN) supplied demographic profiles on active duty personnel identified in the study and the Defense Manpower Agency (DMA) provided yearly Army strengths specific for age and race used to calculate the actual incidence rates of testicular tumor within the Army.

To date 181 cases of testicular tumor have been identified in active Army personnel during the period 1 Jan 72 to 31 Dec 76. Of this total 171 occurred in Caucasian males and 7 occurred in Blacks. The race of the remaining 3 individuals could not be determined. According to data from DMA, Caucasian males served a total of 3,007, 566 man years\* in the Army during 1972-76 while Blacks served a total of 690,955 man years during the same period. Using these data an incidence rate of 5.69 cases of testicular tumor per 100,000 Caucasian males was calculated. The rate among Black males was found to be 1.01 cases per 100,000.

A major objective of this study was to determine if a certain subgroup within the Army has experienced an increased incidence rate of testicular tumor. Concern had developed that certain occupational exposures might be associated with an increased rate of this disease. However, a thorough analysis of the findings of this study has failed to demonstrate a statistically significant increase in the rate of testicular tumor in this subgroup.

---

\* Sum of the mid year populations for the five years

## 5. Renal Disease In Active Duty Military Personnel

In 1976, a joint NIH/WRAIR study of the incidence and prevalence of hospitalization for genito urinary (GU) disease in the Active Duty Military was undertaken. A 300 character, computer based discharge summary (I.P.D.S.) was requested on all personnel whose diagnostic codes were identified as probable GU disease. Earlier record retrieval problems were overcome and currently all GU records from 1 Jan 1971 to 30 June 1977 are available.

Selected diagnostic codes for the years 1971-1973 were compared against U. S. Air Force and U. S. Navy records and in general were shown to be comparable. Mid year strengths by age, race, sex and rank have been received from the Defense Manpower Agency (DMA) for calculation of rates once the numerators are derived. Work currently in progress includes:

(a) retrieval of all GU codes of U. S. Army personnel hospitalized in USAF and USN facilities.

(b) condensation and verification of the records from IPDS. Records on all retired, dependent and reserve beneficiaries will be discarded.

(c) tabulation of all renal records from the Walter Reed Army Medical Center for the years 1972-1976 as a control for record quality.

Following the above, all cases of GU disease in Active Duty U. S. Army personnel will be tabulated for the study years by age, race and sex. Rates will then be calculated using strength figures from the DMA. As a final step, selected diagnostic categories will be sampled for accuracy of diagnoses.

## 6. Possible Association Of Leukemia With 1957 Nuclear Weapon Testing

In late 1976 acute myelogenous leukemia was diagnosed in an individual who had participated in an atmospheric nuclear weapon test in 1957 while on active duty. This raised questions about the possible association between the individual's disease and the earlier radiation exposure. Following news media speculation on this possibility, officials at CDC attempted to determine if there had been any cases of leukemia in the other participants in this test and were able to uncover 9 probable cases. Subsequently a comparison of 3143 names of participants at the 1957 nuclear test (almost all were active Army personnel) with names of leukemia cases in AFIP files revealed 16 more possible cases. Since the expected incidence in the exposed group over 20 years is 2.24 cases (95% confidence, Poisson: 0.46-6.5 cases), EPICON was tasked by TSG to initiate a preliminary study of the incidence of leukemia in participants in nuclear tests.

The EPICON study had 3 objectives: (1) to confirm the diagnosis in the 26 possible cases of leukemia and verify their identity as participants in the 1957 nuclear test, (2) to initiate a broad retrieval of leukemia cases from available data bases to identify additional cases in participants in this test, (3) to assess the data available and advise whatever agency is subsequently designated to perform a definitive study.

Possible cases were validated through Military and V. A. records. Of the 26 possible cases 6 were confirmed, 19 were rejected, and 1 remains indeterminate. A case was rejected if the diagnosis proved not to be leukemia or the identity of the case did not match that of the individual with the same name on the list of participants in the nuclear test.

Approximately 500 leukemia cases have been identified from the IMR/IPDS data base and from the records of various military medical treatment facilities. None of these cases has been identified as a participant in the 1957 test.

Since ascertainment of the complete medical history for a group of men on active duty in 1957 is not possible, we cannot know if all leukemia cases in this group have been identified. However, the readily available data bases within the military are not likely to yield additional cases. If additional cases are found they will likely come from civilian sources. For this reason an agency such as the Medical Followup Agency of the NRC is better suited to perform a definitive study of this group than is EPICON.

If and when an agency is designated by DA or DOD to perform the definitive study, data collected by EPICON to date will be made available to that agency.

#### 7. Swine Influenza A at Fort Dix, New Jersey

Investigation of the swine influenza A outbreak at Fort Dix, New Jersey, in January-February 1976 has been completed. Four reports dealing with this investigation have been accepted for publication in the scientific literature and are listed at Literature Cited.

#### 8. Prevalence of Antibody to Seven Adenovirus Types in Basic Combat Trainees

In early 1976 a study was initiated to obtain current data on the prevalence of antibody to several adenovirus types in trainees arriving at basic combat training (BCT) centers. These data were required to estimate the potential impact of adenovirus epidemics on BCT posts and to provide a basis for the design of adenovirus type 21 vaccine trials.

Sera for the determination of neutralizing antibody (N antibody) were obtained from four different BCT centers using two different sampling methods. At Fort Dix, during a four day period in February 1976, 49 of 550 men undergoing medical inprocessing were identified for the collection of sera in conjunction with the swine influenza A investigation (trainees selected had a terminal "9" in their Social Security Number). Specimens were obtained from 47 men and, after completion of the influenza serologic studies, all 47 specimens were included in this study.

At Forts Jackson, Knox, and Leonard Wood, during 22-24 March 1976, all trainees undergoing medical inprocessing with a terminal "1", "6", or "9" in their Social Security Number (444 men) were identified for the collection of a blood specimen. Specimens were obtained from 423 trainees (Table 1) who had

never been through basic training. (Ten soldiers identified for study at Fort Jackson and four identified for study at Fort Knox were not bled because of prior service.)

At the time specimens were obtained at Forts Jackson, Knox, and Leonard Wood, a questionnaire was administered and data on age, education, residence, and other variables were obtained.

Prevalence values for N antibody titers 1:2 to seven adenovirus types are presented in Table 2 (lack of sera and/or human embryonic kidney cell types). These values are not uniformly similar for all posts. For example, the prevalence of titers 1:2 to type 7 for Fort Leonard Wood trainees is significantly different from that observed for Fort Knox trainees ( $\chi^2$  corrected = 4.55, p 0.05). The prevalence values for titers 1:2 to type 3 for Fort Leonard Wood trainees and for Fort Jackson trainees also are significantly different ( $\chi^2$  corrected = 5.83, p 0.025).

Associations between seropositivity and variables included on the questionnaire administered are being determined. If seropositivity is related to one or more demographic variables, the epidemic potential for adenovirus outbreaks may be different for different BCT centers and may vary over time.

Table 1

Numbers of Basic Trainees Medically Inprocessed, Numbers Selected for Study, and Numbers and Percents Studied for Antibody to Several Adenovirus Type at Three Training Centers (1976)

Fort	Inprocessed no.	Selected for study no.	Studied no.	%*
Jackson	515	163	153	29.7
Knox	487	145	135	27.7
L. Wood	537	136	135	25.1

\* Percent of no. inprocessed.

Table 2

Prevalence of Neutralizing Antibody Titers  $\geq 1:2$  to Seven Adenovirus Types in Basic Combat Trainees (1976)

Fort	Trainees studied	Percent with titer $\geq 1:2$ to:						
		Type 3	Type 4	Type 7	Type 11	Type 14	Type 16	Type 21
Dix	47	ND*	19.1	48.9	10.6	2.1	ND*	12.8
Jackson	153	52.3 <sup>+</sup>	28.1	48.4	13.7	5.2	7.3 <sup>+</sup>	12.4
Knox	135	ND*	20.0	44.4	12.6	ND*	ND*	9.6
L. Wood	135	70.1 <sup>#</sup>	16.3	31.1	6.7	6.0 <sup>#</sup>	2.2 <sup>+</sup>	12.6

\*ND = Not done.

+ Specimens from two trainees were not studied.

# The specimen from one trainee was not studied.

#### 9. Absence of Antibody to Poliovirus Types in Basic Combat Trainees

In March 1977 the Department of Virus Diseases was tasked to present to the Armed Forces Epidemiology Board current data on the lack of antibody to poliovirus types in new basic combat trainees. These data were required to make decisions relating to the immunization of trainees against polioviruses. Time did not permit development and execution of a new study, so sets of stored sera were reviewed to identify specimens for the determination of neutralizing antibody (N antibody) to the three poliovirus types.

The following sera were tested: 47 specimens collected from Fort Dix trainees undergoing medical inprocessing in February 1976 in conjunction with the swine influenza A investigation; and, sera obtained in March 1976 in conjunction with the study of antibody to adenovirus types in basic trainees (see Prevalence of Antibody to Seven Adenovirus Types in Basic Combat Trainees, 1976-1977 WRAIR Annual Report, Work Unit 001). In the adenovirus antibody prevalence study trainees from Forts Jackson, Knox, and Leonard Wood had been bled. Sera from Fort Knox trainees could not be used since these specimens had been split for other studies and quantities remaining were insufficient for testing. All 47 specimens from Fort Dix, 76% of those collected at Fort Jackson, and 77% of those collected at Fort Wood were tested for antibody to all three poliovirus types (Table 1). Insufficient serum prevented testing of some of the Wood and Jackson specimens.

Percents of trainees lacking antibody (N antibody titer 1:4) to poliovirus types are presented in Table 2; 21.2% of Fort Dix trainees, 17.9% of Fort Jackson trainees, and 24.0% of Fort Wood trainees lacked antibody to one or more types. Using data obtained by administration of a questionnaire at the time specimens were collected at Forts Jackson and Wood, lack of antibody to one or more poliovirus types was related to several demographic variables.

Data relating lack of antibody to age are presented in Table 3. Conclusions cannot be drawn regarding the 22-35 year age group since a small number of people were scattered over a wide age range. Also, data for the other age groups must be interpreted with caution because of the small numbers of people studied. These data do suggest that trainees in the 17 year old age category represent a distinct population with regard to poliovirus antibody seropositivity.

Data on lack of antibody and formal schooling completed (years of full time enrollment in grade school, high school, college, professional school, and technical school) are given in Table 4. A trend is not apparent and differences observed could be related to age.

Trainees at Forts Jackson and Wood were asked the city or town and the state where they had lived the longest prior to their eighteenth birthday (childhood residence). Of those studied, 212 gave a childhood residence within the 50 United States or the District of Columbia. Cities and towns were coded according to populations obtained in 1970 censuses, or according to populations obtained in later special censuses (1975 World Almanac and Book of Facts, Newspaper Enterprise Association, Inc., New York, pp. 159-183: source of data; U.S. Bureau of the Census). States were classified according to geographic region.

Data comparing absence of antibody and population of childhood residence are given in Table 5. Using 5,000 and 5,000 to define urban and non-urban residences, 21% of trainees from urban areas and 19.0% from nonurban areas lacked antibody to one or more poliovirus types. Additionally, no trends with regard to population of childhood residence were apparent.

When absence of antibody was compared to geographic region of childhood residence (Table 6), striking differences were apparent. Thirty-six percent of trainees from the northeast, compared to only 9% of trainees from the west, lacked antibody to one or more types. It should be noted that few people were studied from the New England and Mountain states.

The findings reported here should not be interpreted as conclusive evidence that certain demographic variables are related to the absence of antibody to poliovirus types. Samples are small and analyses of data are incomplete. However, data presented do suggest that the proportion of incoming U.S. Army basic combat trainees lacking antibody to one or more poliovirus types may vary, depending upon the characteristics of the populations from which they came. (This study is complementary to work described under DA OA 6441, Work Unit 130, entitled "Viral Infections of Man.")

Table 1

Numbers of Trainees Bled for Original Studies and Numbers and Percents Studied for Antibodies to Three Poliovirus Types

Fort	Bled for original study no.	Studied for <u>poliovirus antibodies</u>	
		no.	% <sup>+</sup>
Dix	47	47	100.0%
Jackson	153	117	76.5
Knox	135	0	0.0
L. Wood	135	104	77.0

<sup>+</sup> Percent of no. bled for original study.

Table 2

Basic Combat Trainees Lacking Neutralizing Antibody (titer <1:4) to Poliovirus  
(1976)

Fort	Studied no.	Percent lacking antibody to:					
		Type 1	Type 2	Type 3	All three Types	Two Types	One Type
Dix	47	6.4%	10.6%	10.6%	2.1%	2.1%	17.0%
Jackson	117	6.8	8.5	9.4	0.8	5.1	12.0
L. Wood	104	10.6	7.7	14.4	1.0	6.7	16.3

Table 3

Absence of Neutralizing Antibody (Titer  $\leq 1:4$ ) to One or More  
 Poliovirus Types by Age  
 (Forts Jackson and L. Wood Basic Combat Trainees)

Age <sup>+</sup> (yrs.)	No. in group	Lacking Antibody to One or More Types	
		no.	%
17	49	16	32.6%
18	60	12	20.0
19	37	4	10.8
20	27	4	14.8
21	23	4	17.4
22-35	25	6	24.0
All ages	221	46	20.8%

<sup>+</sup> Trainees were 17 to 35 years old.

Table 4

Absence of Neutralizing Antibody (Titer <1:4) to One or More Poliovirus Types by Years of Formal Schooling Completed (Forts Jackson and L. Wood Basic Combat Trainees)

Formal schooling completed (yrs.)	No. in group	Lacking antibody to one or more types	
		no.	%
≤ 9	36	10	27.8%
10	44	7	15.9
11	48	8	16.7
12	77	18	23.4
≥ 13	16	3	18.8
All groups	221	46	20.8%

Table 5

Absence of Neutralizing Antibody (Titer <1:4) to One or More Poliovirus Types by Population of Place of Childhood Residence (Forts Jackson and L. Wood Basic Combat Trainees)

Population of childhood residence	No. in group	Lacking antibody to one or more types	
		no.	%
<5,000	63	12	19.0%
≥5,000	149	31	20.8%
5,000-24,999	38	10	26.3
25,000-99,999	33	5	15.2
100,000-499,999	37	9	24.3
≥500,000	41	7	17.1
All groups	212	43	20.3%

Table 6

Absence of Neutralizing Antibody (Titer <1:4) to One or More Poliovirus Types by Geographic Region of Childhood Residence (Forts Jackson and L. Wood Basic Combat Trainees)

U.S. geographic region <sup>+</sup>	No. in group	Lacking antibody to one or more types	
		no.	%
<u>NORTHEAST</u>	33	12	36.4%
New England	5	2	40.0
Mid. Atlantic	28	10	35.7
<u>NORTH CENTRAL</u>	63	14	22.2%
E. N. Central	47	10	21.3
W. N. Central	16	4	25.0
<u>SOUTH</u>	70	13	18.6%
S. Atlantic	35	5	14.3
E. S. Central	15	3	20.0
W. S. Central	20	5	25.0
<u>WEST</u>	46	4	8.7%
Mountain	7	2	28.6
Pacific	39	2	5.1
All U.S. regions	212	43	20.3%

<sup>+</sup> Center for Disease Control, Morbidity and Mortality Weekly Report Annual Summary 1976. Aug. 1977: vol. 25, no. 53.

10. Simultaneous Administration of Live, Enteric-Coated, Oral Adenovirus Types 4, 7, and 21 Vaccines: Safety and Immunologic Response

After the occurrence of adenovirus type 21 (ADV-21) acute respiratory disease in basic combat trainees immunized with adenovirus type 4 (ADV-4) and adenovirus type 7 (ADV-7) vaccines in 1975, the Surgeon General of the Army directed further testing of an ADV-21 vaccine which had been tested previously for safety and immunogenicity in 45 volunteers. In August 1976 a study was undertaken at Lackland Air Force Base, Texas, to determine the safety of and the immune response to ADV-4, ADV-7, and ADV-21 vaccines given simultaneously to human volunteers.

Utilizing a double blind study design, 476 volunteers were randomly placed into four vaccine groups and received either: ADV-4, ADV-7, and ADV-21 vaccines (4/7/21 group); ADV-4 and ADV-7 vaccines with a placebo tablet (4/7/p group); ADV-21 vaccine with two placebo tablets (p/p/21 group); or three placebo tablets. Volunteers were followed for 21 days and neutralizing antibody levels to vaccine viruses received were determined. The occurrence of illness was monitored to assess safety and was similar in all study groups. Adenoviruses were not isolated from volunteers hospitalized for acute respiratory disease and low rates of seroconversion to adenoviruses were observed in volunteers not receiving vaccine viruses.

Pre- and postimmunization neutralizing antibody titers were determined only for volunteers who initially lacked antibody (titer less than 1:2) to the vaccine virus or all vaccine viruses they received. The percents of volunteers seroconverting to ADV-4 were similar in the 4/7/21 and 4/7/p groups (78% and 74%, respectively). Seventy-nine percent of 4/7/p volunteers but only 62% of 4/7/21 volunteers developed antibody to ADV-7. Regarding ADV-21 seroconversions, 70% of group p/p/21 and only 58% of group 4/7/21 developed antibody. Both ADV-7 and ADV-21 are in hemagglutination subgroup I while ADV-4 is in subgroup III, suggesting interference may occur between adenoviruses of the same subgroup but not between those less closely related.

A report of this study has been completed and submitted to the Army Investigational Drug Review Board; a manuscript for publication in the scientific literature is in preparation. (This work is complementary to work described under DA OA 6441, Work Unit 130, entitled "Viral Infections of Man.")

11. Simultaneous Administration of Live, Enteric-Coated, Oral Adenovirus Types 4, 7, and 21 Vaccines: Safety, Efficacy, and Immunogenicity

This study was initiated at Fort Dix, New Jersey, in October 1976. Using a double blind study design 600 male volunteers from the basic combat trainee population were randomly assigned to one of two study groups shortly after their arrival on the post. Group 4/7/21 members (300 men) received adenovirus type 4 (ADV-4), adenovirus type 7 (ADV-7), and adenovirus type 21 (ADV-21) vaccines; group 4/7/p volunteers received

ADV-4 and ADV-7 vaccines and a placebo tablet.

Participants had prevaccination and three week post vaccination sera specimens drawn and were followed through basic combat training to assess vaccine safety and efficacy. Hospitalization and outpatient records were reviewed and abstracted and paired sera and a throat swab for virus isolation were obtained from volunteers hospitalized for acute respiratory disease (ARD).

Rates of hospitalization for causes other than ARD were similar for both study groups. ARD hospitalization rates are presented in Table 1. Considering that approximately three weeks must elapse after immunization for the maximum antibody response to occur, ARD rates are shown for the periods < 21 days and ≥ 22 days after administration of vaccine viruses.

Table 1. Hospitalization rates (admissions/100 men) for ARD for the periods ≤ 21 days and ≥ 22 days after immunization.

Study group	Period after immunization			
	≤ 21 days		≥ 22 days	
	No. admissions	Rate*	No. admissions	Rate†
4/7/21	19	6.3	13	4.4
4/7/p	5	1.7	22	7.6

\* Based on 300 men in each group (populations beginning the study).

† Based on 295 men in group 4/7/21 and 289 men in group 4/7/p (study populations at the end of three weeks).

Hospitalized cases of ARD in the 4/7/21 group and in the 4/7/p group were similar when compared with regard to severity of illness and duration of hospitalization. No significant differences were observed when outpatient visits for the two groups were compared.

Adenovirus types other than ADV-21 were isolated from three volunteers hospitalized for ARD. One had an ADV-4 isolate and two had isolates of ADV-7. All three were in the 4/7/21 group and were hospitalized within 21 days after entering the study.

Only one individual, a 4/7/p group member, had ADV-21 isolated from their pharyngeal swab in the first three weeks after entering the study. Numbers of volunteers from whom ADV-21 was isolated during the period ≥ 22 days after immunization are presented in Table 2.

Table 2. ADV-21 isolations from volunteers hospitalized for ARD ≥ 22 days after immunization.

Study group	Volunteers in the group	Volunteers with an ADV-21 isolate	
		No.	%
4/7/21	295	5	1.7
4/7/p	289	11	3.8

The disproportionate number of ARD hospitalizations in the 4/7/21 group during the first three weeks after immunization, at present, has not been explained. Testing of sera for neutralizing antibody to adenovirus types was interrupted by a lack of primary human embryonic kidney cell cultures. Alternative serologic tests currently are being developed. (This work is complementary to work described under DA OA 6441, Work Unit 130, entitled "Viral Infections of Man.")

## 12. Congenital Malformation Rates for Several Army Posts

During the study of an outbreak of viral hepatitis at Fort Hood, Texas, (October 1972-March 1975) data were obtained on congenital malformations in infants born at Darnall Army Hospital (Fort Hood). These data were collected to determine if changes in congenital malformation (CM) rates, related in time to the hepatitis outbreak, had occurred. Preliminary analysis revealed an increase from 1971 to 1972.

Early in 1975 available Individual Patient Data System (IPDS) computer printouts and clinical record cover sheets (CRCSS) for Darnall Army Hospital for the period 1 January 1971-30 June 1974 were reviewed at the Walter Reed Army Institute of Research. CM rates/1,000 total births were determined for all malformations, major malformations, and minor malformations using Center for Disease Control (CDC) criteria. Increases in rates from 1971 to 1972 were observed for all three categories, but these data were hand tabulated from IPDS printouts which were not designed to facilitate counting of individual patients and were therefore considered provisional. A more extensive study of CMs at Fort Hood was recommended.

The Chief of the Epidemiology Consultant Service (EPICON) directed the investigation to commence by determining CM rates over several years for Fort Hood and other similar Army installations using IPDS data. Subsequent studies, if necessary, would be developed following this initial evaluation. IPDS data, stored on computer tape at the U.S. Army Health Information Systems and Biostatistical Agency (Fort Sam Houston, Texas), were received in March 1976.

Posts selected for study, in addition to Fort Hood, were: Forts Campbell and Knox, Kentucky; Fort Carson, Colorado; Fort Riley, Kansas; Fort Sill, Oklahoma; and, Fort Rucker, Alabama. The comparison posts were selected because they were similar to Fort Hood in that they had large numbers of births but did not have training programs in pediatrics, obstetrics and gynecology, or family practice. Large study populations were desirable since CMs occur infrequently. Training programs were considered since it was thought that diagnostic awareness and/or capability might differ between teaching and non-teaching hospitals.

The study populations consisted of infants identified by IPDS as having been born at one of the selected posts from 1 January 1971 through 30 June 1975. The IPDS record contains 300 characters in 76 fields and includes several demographic variables and International Classification of Diseases,

eight revision, (ICDA-8) diagnostic codes. These data are generated from CRCS information upon discharge of a patient. Only records of infants whose age and diagnostic birth codes were compatible and indicated the record had been generated in conjunction with a birth admission at a selected medical facility were studied.

Records for 40,638 infants were received. Twenty-nine records were excluded because of incompatible age and diagnostic birth codes (e.g., age code indicated liveborn infant and diagnostic birth code indicated stillborn.) Data from 1 January-30 June 1975 were incomplete since records of all infants born prior to or on 30 June 1975 but discharged on or after 1 July 1975 were not included on the IPDS tapes studied.

Excluded from the study were infants born in civilian institutions under the Civilian Health and Medical Program of the Uniformed Services (CHAMPUS). Records for these patients were not obtained because EPICON did not have access to CHAMPUS records.

Only congenital anomalies in liveborn infants were studied. Initially, this investigation was to include the study of CMs in both liveborn and stillborn infants. However, in January 1976 a telephone survey of pathologists and obstetricians at the seven selected installations was conducted to determine criteria used for stillbirths. Criteria varied and physicians at some installations reported the criteria were not strictly followed.

Congenital anomalies are covered by ICDA-8 rubrics 740-759.9. Since this study was limited to the generation of rates using coded discharge diagnoses recorded for the hospital admission associated with birth, several factors were considered:

1. Some CMs are of little or no medical or cosmetic significance. Therefore, uniformity in recording these types of CMs must be questioned.
2. Some CMs may not be identified easily by a general pediatrician during the hospital admission associated with birth.
3. Some discharge diagnoses tend to be incorrectly coded. For example, during an earlier investigation, it was found that a discharge diagnosis of "murmur" often was coded as a heart anomaly, and a discharge diagnosis of "hip click" was coded frequently as congenital dislocation of the hip.
4. And, some ICDA rubrics are broad and cover anomalies of both major and minor significance.

Taking the above factors into account, the ICDA rubrics were divided into the following three categories:

1. Category I: Rubrics in this category are, for the most part, associated with CMs of little or no medical or cosmetic significance (minor CMs).

2. Category II: Contains rubrics meeting one or more of the following criteria:

a. The scope of the rubric covers both major (serious) and minor malformations.

b. The rubric is associated with a major CM which is not easily identified by a general pediatrician at birth.

c. The rubric contains one or more diagnoses of major CMs which may be incorrectly coded.

3. Category III: Contains rubrics meeting all of the following criteria:

a. The scope of the rubric covers only major malformations which would tend to be recorded uniformly.

b. The malformations covered by the rubric are considered easily identifiable by a general pediatrician on a newborn physical examination.

c. The rubric contains malformations which are unlikely to be incorrectly coded.

Classification of the rubrics into the above three groups was done by LTC Richard D. Landes, MC (Pediatric Neonatologist, Walter Reed General Hospital).

Determination of rates for each of the three CM categories by six month period, for each of the seven installations, is near completion. For categories II and III, rates are being generated according to the organ system involved.

### 13. Possible Association of Guillain Barre Syndrome with Influenza Immunizations

During 1976 a massive influenza immunization campaign was undertaken in the United States in an effort to counteract a possible epidemic of swine influenza. This campaign was abruptly halted in December 1976 because of an apparent increased incidence of Guillain-Barre Syndrome (GBS) associated with immunizations. DOD also stopped influenza immunizations within the military on the recommendation of the USPHS.

Because the US military services have had extensive experience with influenza immunizations, the 3 Surgeons General were requested by the USPHS for any data they may have on the association of GBS with immunization. The Surgeon General of the Army in turn tasked EPICON with the mission of generating this data and providing an analysis.

Since vaccination records are hard to access on a large scale and their accuracy uncertain, another index of vaccination was needed. Since almost all cases of GBS in the civilian experience occurred within 10 weeks

of vaccination and essentially all military influenza vaccinations (except in recruits) are given in October, the incidence of GBS for the years 1971-1976 was examined for temporal clustering in the period following vaccination (Oct-Jan). In addition, since the CDC criteria for a case was rather simple (objective motor weakness and a physician's diagnosis of GBS), a comparison was made with the more stringent criteria of Osler and Sidell for validation (1).

Casefinding relied on the automated Individual Patient Data System (IPDS) record. GBS (and several other neurologic syndromes) are coded as "3540" in ICD-8 nomenclature. All records with this code plus 3 related codes were identified through IPDS. A review of Clinical Record Cover Sheets (CRCS) in all four categories confirmed GBS cases were only coded as "3540". The hospital records were then requested for all 127 individuals identified by IPDS with "3540". Strength data to allow computation of rates was supplied by the DOD manpower information office. To date 113 (89%) of these hospitalization records have been found and reviewed. 79 cases (excluding 18 recruit cases) would be classified as GBS by CDC criteria. 51 cases (excluding 15 recruits) would also meet the Osler-Sidell criteria. No cases met only the Osler-Sidell criteria and not CDC.

When the recruit and non-recruit cases were temporarily arranged the first quarter of the year has a greater number of cases in both groups but does not significantly deviate from a random distribution by month (Edwards Test for seasonality:  $\chi^2 = 1.04$  with 2 df). The 14 unretrieved records were uniformly distributed through the year with no more than two hospitalizations occurring in any one month. No increase in cases in the final quarter of the year was apparent. Only two of the eight January non-recruit cases were hospitalized prior to the 15th of the month.

Rates of GBS per 100,000 population are shown on Table 1 by the CDC criteria. Generally, observed rates are between one and three cases per 100,000 population per year with approximately one-third of CDC criteria positive cases being unable to meet the Osler-Sidell criteria. A comparison of the Army rate for GBS by quarter and CDC's calculation of national rates shows the Army rates fall intermediate between CDC's vaccine associated and non-vaccine associated rate of disease. Recruits do not appear to have a risk of GBS greater than that of their non-recruit age contemporaries with a proportion of 1.41 cases of GBS out of each 100,000 enlisted inductees.

Table 1. Rates of GBS by CDC Criteria\* According to Quarter of Hospital Admission

JAN-MAR	APR-JUN	JUL-SEP	OCT-DEC
2.93	1.62	2.12	1.73
[CDC: Oct-Dec 76 Vaccinees (> 17 Years of Age) 10.92 Non-vaccinees (> 17 Years of Age) 1.12]			

\*Rates expressed as cases/100,000 men/Year

As shown in Table 1, the rate of occurrence of GBS during the October-December quarter, which would be expected to reflect a flu vaccine associated increase in GBS, is one of the lower rates. The higher rate in the January-March quarter probably reflects the seasonal increase in respiratory infections.

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Task 00 Military Preventive Medicine

Work Unit 001 Epidemiologic studies of military diseases

Literature Cited.

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SECY*	6. WORK SECURITY*	DA 0A 6445	77 10 01	DD-DR&E(AR)636
76 10 01	D. Change	U	U	NA	NL	8d. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO. CODES*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	9. LEVEL OF SUM A. WORK UNIT	
a. PRIMARY	62770A	3M762770A802		00	WORK UNIT NUMBER 002	
b. CONTRIBUTING						
DESOXOXXXX	CARDS 114F					
11. TITLE (Precede with Security Classification Code)* <u>(U) Pathogenesis of Enteric Diseases</u>						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS* 010100 Microbiology						
13. START DATE 59 05	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-house		
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE PRECEDING		19. PROFESSIONAL MAN YRS		20. FUNDS (in thousands)
a. DATES/EFFECTIVE: NA	EXPIRATION:	FISCAL YEAR	77	3	373	
b. NUMBER:		CURRENT	78	5	447	
c. TYPE:	d. AMOUNT:					
e. KIND OF AWARD:	f. CUM. AMT.					
21. RESPONSIBLE DOD ORGANIZATION		22. PERFORMING ORGANIZATION				
NAME* Walter Reed Army Institute of Research		NAME* Walter Reed Army Institute of Research Div of CD&I				
ADDRESS* Washington, DC 20012		ADDRESS* Washington, DC 20012				
RESPONSIBLE INDIVIDUAL RAPMUND, COL G.		PRINCIPAL INVESTIGATOR (Punish SBN if U.S. Academic Institution)				
NAME: TELEPHONE: 202-576-3551		NAME: FORMAL, S. B. TELEPHONE: 202-576-3344 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]				
23. GENERAL USE Foreign intelligence not considered		ASSOCIATE INVESTIGATORS NAME: GEMSKI, P. NAME: KEREN, D., O'BRIEN, A.				
24. KEYWORDS (Precede EACH with Security Classification Code) (U) Diarrhea; (U) Dysentery; (U) Bacillary; (U) Salmonellosis; (U) Immunity; (U) Immunization						
25. TECHNICAL OBJECTIVE, 26. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number. Precede rest of each with Security Classification Code)						
23. (U) To study the pathogenesis of bacterial infections of the gastrointestinal tract, particularly those caused by Shigella, Salmonella and Escherichia coli is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised. Diarrhea is a significant problem in military personnel operating overseas.						
24. (U) The genetic control of O-antigen specificity of enteric pathogens is being studied since such cell envelope components are of importance in disease and its prevention through vaccination.						
25. (U) 76 10-77 09 A Shigella dysenteriae-like toxin has been extracted from an Escherichia coli strain responsible for diarrhea in infant rabbits. A similar toxin has been obtained from strains of E. coli associated with epidemics of diarrhea in human feces. Two inbred mouse strains which express B lymphocyte immune defects were 1000 times more susceptible to Salmonella typhimurium infection than syngeneic controls. The local immune response to the intestine to Shigella is being studied in order to establish the optimal route, dosage and vaccine for stimulating effective protection against these organisms. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.						
129						

\*Available to contractors upon prime contractor's approval

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1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65  
AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Work Unit 002 Pathogenesis of Enteric Diseases

Investigators

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Description

The pathogenesis of bacterial infections of the gastrointestinal tract, particularly those caused by Shigella, Salmonella and Escherichia coli is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised.

Progress

By employing an integrated, immunologic, cytologic and genetic approach (see previous annual reports) studies in this department are concentrating on further elucidation of: (I) virulence factors and mechanisms involved in intestinal penetration and toxin elaboration by pathogens, key mechanisms by which enteric diseases are provoked; (II) the genetic control of O antigen specificity of enteric pathogens since such cell envelope components are of importance both in disease and its prevention through vaccination; and (III) the application of genetic techniques for development of live, oral vaccines against Shigellosis.

1. A Shigella dysenteriae-like toxin has been extracted from an Escherichia coli strain responsible for diarrhea in infant rabbits. A similar toxin has been obtained from strains of E. coli associated with epidemics of diarrhea in human infants.

2. Two inbred mouse strains which express B lymphocyte immune defects were 1000 times more susceptible to Salmonella typhimurium infection than syngeneic controls.

3. The local immune response of the intestine to shigella is being studied in order to establish the optimal route, dosage and vaccine for stimulating effective protection against these organisms.

4. The Vir plasmid of Escherichia coli has been genetically and physically characterized and shown to belong to incompatibility group F111 and to be 84 magadaltons in size.

5. Genetic studies of the heat labile mouse toxin associated with E. coli from bacteremia cases have failed to identify a plasmid mechanism of control.

6. Shigella sonnei hybrids which express E. coli antigen 025 have been shown to retain their ability to synthesize native phase I antigen. Preliminary findings on the genetic control of antigenic phase variation of S. sonnei which occurs on a probability of  $10^{-2}$  per bacterial division suggest a plasmid recombinational event.

7. Continuing studies of converting phage Sf6 have shown that it is a generalized transducing phage, morphologically similar to the C phages of the Bradley scheme. Preliminary studies indicate that Sf6 possesses endorhamnosidase activity.

8. Smooth 025 and 08 hybrids of E. coli K12 containing various virulence factors have been constructed.

#### Materials and Methods, and Results in 8 Areas of Research Named Above

1. E. coli strains associated with diarrheal disease in man and animals are believed to cause disease by one of two mechanisms. Some strains invade the intestinal mucosa while other strains elaborate a cholera toxin-like heat-labile and/or heat stable enterotoxin. However, a non-invasive, non-enterotoxin producing strain of E. coli, which we designate South Carolina (SC), was recently described which causes diarrhea and death when fed to young rabbits. We postulated that this strain which adheres to the intestinal mucosa may cause diarrhea by production of an S. dysenteriae (shiga)-like toxin. Therefore, French press extracts of strain S.C. grown for 72 hours in a modified syncase broth were partially purified according to the same procedure used for shiga toxin production. Extracts were precipitated with  $(\text{NH}_4)_2\text{SO}_4$ , chromatographed on DEAE Sephadex A-50, and the eluate subjected to gel filtration on Biogel A 0.5M. Such preparations, like shiga toxin, were cytotoxic for Hela cells and enterotoxic for rabbit ileal loops. Cytotoxicity was neutralized by both shiga and S. flexneri antitoxins. Similar results were obtained with four strains of E. coli with classical enteropathogenic serotypes which were isolated during epidemics of diarrhea among human infants. Serum samples obtained from such infants will be examined for neutralizing antibody to the shiga-like E. coli toxin to determine if this toxin is produced in vivo.

2. Resistance to S. typhimurium infection in eight inbred mouse strains has been shown to be influenced by a single dominant autosomal gene. The gene products (T lymphocytes, B lymphocytes, macrophages) responsible for this natural immunity are unknown. Theoretically, if one could selectively transfer various cell types between syngeneic mouse

strain which differ in susceptibility to the organism, one could determine which cell types are responsible for natural immunity to S. typhimurium. To assess possible models for such transfer studies, the LD<sub>50</sub> of S. typhimurium (strain TML) administered intraperitoneally was determined for various inbred mouse strains and histocompatible (syngeneic) substrains. Two strains with well characterized B lymphocyte immune defects were found which were 1000 times more susceptible to S. typhimurium infection than syngeneic controls: the C<sub>3</sub>H/HeJ and CBA/N strains. The C<sub>3</sub>H/HeJ strain is unresponsive to the lethal and mitogenic effects of LPS. The CBA/N strain has an X-linked B lymphocyte immune defect-expressed as a subnormal antibody response to LPS, a poor B cell mitogenic response to such T independent antigens as LPS, and a deficient antibody dependent cellular cytotoxicity response. We attempted to transfer natural immunity to S. typhimurium with spleen cells and bone marrow cells from C<sub>3</sub>H/HeN (immunologically normal, S. typhimurium resistant) to C<sub>3</sub>H/HeJ mice and from CBA/NXDBA/2N F<sub>1</sub> females (immunologically normal, S. typhimurium resistant) to CBA/NSDBA/2N F<sub>1</sub> males (immunologically abnormal, S. typhimurium sensitive). Spleen cell transfers did not confer protection against S. typhimurium in either model. However, bone marrow transferred from CBA/NXDBA/2N F<sub>1</sub> females conferred protection against S. typhimurium to F<sub>1</sub> males. In addition, we established an association between the genes expressing the immunological defect in the CBA/N animals and sensitivity to S. typhimurium. These studies indicate that there is a second gene in addition to the single dominant autosomal gene which influences natural immunity to S. typhimurium in mice.

3. A chronically isolated ileal (Thiry-Vella) loop model is being used to study the immune response of rabbits to candidate dysentery vaccines. This enables us to study the kinetics of the response in addition to adverse effects. Each isolated loop was immunized with  $1 \times 10^8$  cells of live Shigella X16 (a hybrid of Shigella flexneri 2a and Escherichia coli). Secretions from the isolated loops were collected daily. Serum samples (to study systemic humoral immunity) were obtained weekly. Sera and loop secretions were assayed for IgA and IgG antibodies against Shigella X16 using the enzyme-linked immunosorbent assay technique. Each animal received three doses of Shigella X16 -  $10^8$  organisms on days 1, 8 and 15 after surgery in the isolated loops.

By day 4 after the first dose of Shigella X16, IgA anti-X16 was usually detectable (Figure 1). The IgA anti-X16 content in loop fluids from these animals reached a high level on day 11, decreased on day 14 and thereafter increased through day 21.

The time of appearance and early strength of the IgA response seemed to depend on whether or not a Peyer's patch (aggregates of lymph follicles in the intestine) was included in the isolated loop (Figure 1). Loops lacking a Peyer's patch had a detectable IgA anti-X16 response by day 7, however, these responses rose to levels that were comparable to those found in animals containing a Peyer's patch in their loops by day 18.

Although IgG anti-X16 was not detectable in any loop fluid from either group of rabbits, we have subsequently found that IgG is not stable in these intestinal secretions. Whereas no IgA anti-X16 was found in serum from either group of animals, small amounts of IgG anti-X16 were detected in serum by day 21 in a few rabbits (irrespective of the presence of a Peyer's patch).

Those animals followed for long periods of time were found to lose their local IgA anti-X16 about two weeks after the last intra loop immunization.

The anamnestic response and the effects of oral or subcutaneous priming with the organism are currently being studied with this model.

4. The virulence plasmid (Vir), discovered in an E. coli strain causing bacteremia in a lamb and thought to control the synthesis of a toxin lethal for chickens, mice and rabbits has been characterized. The self-transmissible nature of Vir is evidenced by its high rate of conjugal transfer to several genera of the family Enterobactereaceae. Initial crosses concerned the construction of a Vir transconjugant in the plasmid-free recipient strain, E. coli K12C600 nal<sup>R</sup>. After mating with E. coli donor H209K-Vir<sup>+</sup>, single colony isolates of E. coli C600 nal<sup>R</sup> were tested for inheritance of the Vir plasmid by testing sonicates of such clones for production of toxin lethal to mice. Of 24 clones examined, 19 were found to have inherited Vir. Unlike E. coli strains S5 and H209K - Vir<sup>+</sup> which appeared stable for Vir, E. coli K12 C600 nal<sup>R</sup> Vir<sup>+</sup> transconjugants were found to segregate the Vir plasmid spontaneously at a high frequency. One of these, designated C600 nal<sup>R</sup> Vir<sup>+</sup> 5 and subsequently used in studies of the molecular characterization of Vir, was found to be relatively stable for Vir. When sonicates of 24 independent clones of Vir<sup>+</sup> 5 were tested for lethality to mice, only 2 clones were found to have lost the ability to produce the Vir toxin.

The finding that strains with Vir are sensitive to the F-specific phages suggested that this plasmid belongs to one of the F-incompatibility groups. Crosses were performed therefore between donor strains carrying plasmids representative of incompatibility groups F<sub>I</sub>, F<sub>II</sub>, F<sub>III</sub>, F<sub>IV</sub> and F<sub>V</sub> and recipient strains H209K and C600 nal<sup>R</sup> with and without the Vir plasmid. The analysis of coexistence of Vir with these F plasmids was performed on transconjugants of H209 Vir<sup>+</sup>, because previous observation revealed that Vir was unstable in the E. coli C600 nal<sup>R</sup> strain. The results indicate that the Vir plasmid can stably coexist in E. coli with plasmids of incompatibility group F<sub>I</sub>, F<sub>II</sub>, F<sub>IV</sub> and F<sub>V</sub>. These crosses also revealed no evidence of surface exclusion between Vir and plasmids of the F<sub>I</sub>, F<sub>II</sub>, F<sub>IV</sub> and F<sub>V</sub> incompatibility groups. No significant differences in the frequency of transfer of these plasmids into recipient strains C600 nal<sup>R</sup> and H209K- with and without Vir was observed.

Preliminary findings indicate that Vir cannot coexist with the Col B plasmid which belongs to incompatibility group F<sub>III</sub>. After Col B was conjugally transferred to a E. coli 09 Vir<sup>+</sup> recipient, 30 Col B clones were tested for the presence of Vir. 29 of these has lost Vir as evidenced by failure to make the mouse lethal toxin.

Plasmid DNA was isolated from CsCl-EtBr isopycnic gradients containing lysates of E. coli strains H209 and H209 Vir<sup>+</sup> and examined by means of agarose gel electrophoresis and electron microscopic techniques. The recipient strain H209 was found to contain two cryptic plasmid DNA species (2.2 and < 0.5 megadaltons) whereas the Vir<sup>+</sup> derivative harbored an additional plasmid of 84 megadaltons. The Vir plasmid was transferred via conjugation from E. coli H209 Vir<sup>+</sup> to E. coli K-12 C600. Transconjugants which produced toxin lethal for mice were found to contain the 84 megadalton plasmid species.

5. Some E. coli strains isolated from human sources have been shown to elaborate a heat labile toxin lethal for mice. Such strains are isolated with significantly greater frequency from extraintestinal E. coli infection such as bacteremia, urinary tract infections and neonatal meningitis than from stools of healthy persons (see annual report 1975). Investigation into the genetic control of production of this toxin have been undertaken. A similar toxin produced by E. coli strains isolated from extraintestinal infections in animals has been shown to be under the control of a transmissible plasmid, designated Vir. Thus studies on the genetics of the toxin produced by human isolates initially concentrated on the detection of a similar toxin-controlling plasmid. Genetic studies attempting to transfer conjugally the toxin phenotype or to eliminate it by intercolation were unsuccessful. Physical studies involving agarose gel electrophoresis of restriction endonuclease fragments of plasmid DNA from one such toxin + strain revealed no similarity between plasmid DNA of this toxin producing human isolates and the plasmid controlling toxin production in animal isolates. Experiments investigating a possible chromosomal control of toxin production by human isolates resulted in transfer of almost 50% of the chromosome of toxin producing donor strains to laboratory recipients, without concurrent transfer of the ability to produce toxin. Thus the question of genetic control of production of the heat labile toxin by E. coli isolates from human sources remains open.

6. As reported previously (Ann. report, 1975), the O25 antigen genes of E. coli has been conjugally transferred to S. sonnei phase I (virulent) and S. sonnei phase II (avirulent) recipients. O25 hybrids of the phase I recipient retained their virulence whereas similarly constructed hybrids of the phase II strain failed to regain virulence even though they now elaborate a smooth O side chain. We have considered the possibility that the average number of O25 repeat units of the phase II hybrid was less than that of the phase I hybrid. However, during the past year, it has been revealed that O25 hybrids of the phase I recipient retain their ability

to produce their natural phase I antigen. The presence of this phase I antigen is undetectable by agglutination tests and was established only by means of rabbit immunization and adsorption studies. The presence of phase I antigen in O25 hybrids thus could have a bearing on their virulence.

Studies of the genetic control of phase I instability, i.e. dissociation into phase II, are continuing. Mutational analysis via the zero-point method has established a mutation rate of  $10^{-2}$ /bacterial division. Preliminary screening of DNA extracts from phase I and phase II *S. sonnei* by use of agarose gel electrophoresis has revealed the presence of a small additional plasmid in phase I extracts. It is unlikely that this plasmid is directly involved in synthesis of phase I antigen, since our previous findings indicate that phase I and phase II genes are chromosomal (linked to the *his* and *xyl* loci, respectively). More likely, this plasmid may be involved in the phase variation (I $\rightarrow$ II) of *S. sonnei*. As has been shown for flagella phase variation in salmonellae, we postulate that the phase variation of *S. sonnei* somatic antigens reflects a recombination phenomenon, i.e. insertion of a plasmid into the genes for phase I which causes a "mutational effect", i.e. inability to synthesize antigen I. We are presently introducing the rec A alleles of *E. coli* K12 into wild type *S. sonnei* which would effect recombinational events. If phase variation from I $\rightarrow$ II is affected in such Rec A $^+$  hybrids, physical studies of the plasmid profile of these strains, will be performed.

7. As reported previously (Ann. Report, 1964) a temperate phage, designated Sf6, has been isolated from *Shigella flexneri* 3a. Characterization of Sf6 revealed that it possesses the capacity for converting the *S. flexneri* 3,4 group antigen complex to group factor 6. Serological studies and chemical analysis of lipopolysaccharide from converted strains suggested that group factor 6 is a reflection of an acetylation of the preexisting 3,4 antigen complex. Evidence was provided that the 3,4 group antigen complex functioned at least in part, as a cell surface receptor site for Sf6 adsorption.

During the past year, additional studies of phage Sf6 have been performed. Phage Sf6 has been shown to have the ability for generalized transduction. Sf6 lysates prepared on prototrophic *S. flexneri* Y strain FH10 were shown to transduce chromosomal genes for *arg*, *pro*, and *his* at a frequency of about  $1 \times 10^{-8}$ /pfu.

Thus in addition to the intrinsic value of Sf6 for achieving antigen conversion, this phage, on the basis of preliminary studies, may prove useful as a genetic tool for achieving both intergeneric transduction between *E. coli* and *S. flexneri* and interspecific transduction within the *S. flexneri* group.

Studies on the morphology of phage Sf6 in collaboration with J.A. Wohlhieter (WRAIR) have revealed that Sf6 is typical of the type C phages of the Bradley classification scheme. Electron micrographic examination of the Sf6 phage preparation revealed typical phage particles consisting of a head portion and short tail that are uniform in size. Sf6 phage has a isometric head with hexagonal symmetry 53 nm in diameter. This kind of image indicates that the true shape of the head is probably icosohedral. The tail of the phage, about 16 nm long, appears to contain a bare plate with 3 or more spikes. No contractile sheath was observed.

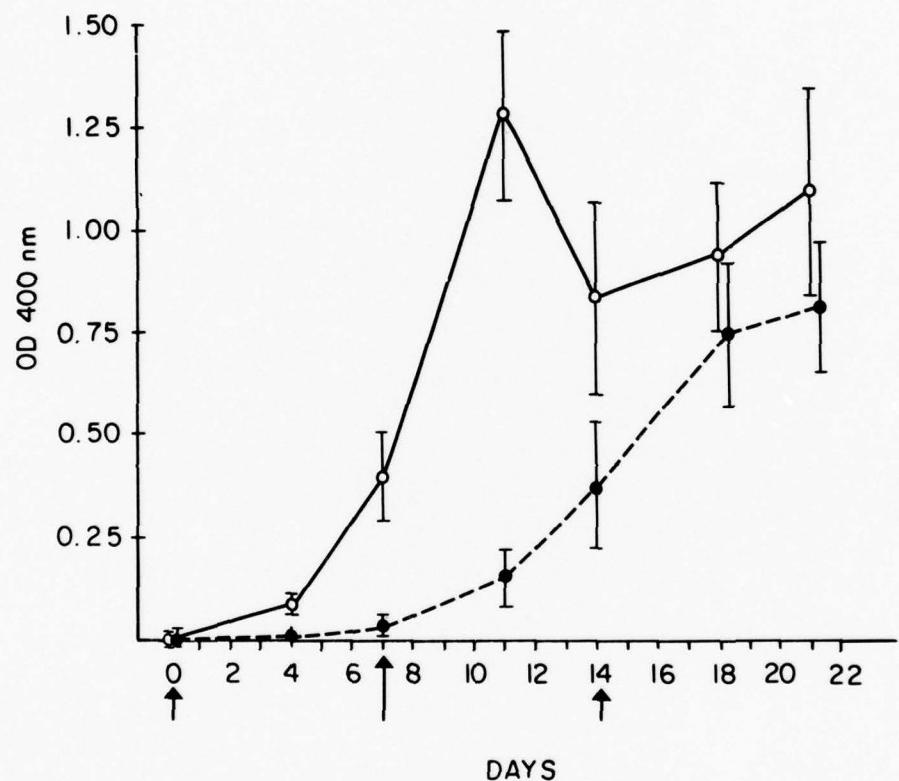
Preliminary studies in conjugation with Dr. A. A. Lindberg (Stockholm) have indicated that phage Sf6 has an endorhamnosidase which cleaves the group 3,4 antigen receptor and that this hydrolysis is a prerequisite for phage Sf6 adsorption. This finding accounts for the previously observed narrow lytic spectrum of phage Sf6. It only lyses S. flexneri which produce only the 3,4 group antigen. The finding that Sf6 phage adsorption is concomitant with the hydrolytic cleavage of the O-polysaccharide chain (3,4 antigen) producing an oligosaccharide of about 4 repeat units ( $\{ \text{GLcNaC-Rha-Rha-RhA} \}_n$ ) is of potential interest in the development of vaccines against shigellosis. Because these oligosaccharides can be readily isolated in preparative amounts by treatment of S. flexneri Y LPS with Sf6 at high phage multiplicity, a new approach (by specific enzymatic cleavage) now exists for preparing lipid free, non-endotoxic O antigens for use in development of synthetic vaccines. Previous efforts to prepare such oligosaccharides by indiscriminate acetic acid cleavage (for removal of Lipid A) failed to yield oligosaccharide antigens of uniform size and therefore of limited potential in preparing synthetic vaccines against shigellosis.

8. E. coli K12 and some of its derivatives have been established (NIH Recombinant Advisory Committee) as the required host vehicle for recombinant DNA cloning by plasmid and phage vector. Of considerable importance in this area of research is the assessment of the risks associated with E. coli K12 as the cloning vehicle. Much of the risks are minimized by the finding that E. coli K12 is unable to persist for long periods of time in the intestine of infected hosts. This failure to colonize is believed to be a consequence, in part, of the genetic defects expressed by K12 in the synthesis of a fully functional lipopolysaccharide layer.

To assess the importance of the LPS in colonization and ability to produce disease, we have genetically transferred genes controlling the synthesis of E. coli 025 and 08 smooth O-antigens into various derivatives of E. coli K12. By conjugation with E. coli Hfr's W3703(025) and RJ88(08), smooth 025 and 08 hybrids of E. coli K12, containing various combinations of the ent plasmid (enterotoxin production), the hly plasmid (hemolysis production) and either the K88 (porcine adhesion factor) or K99 (calf

adhesion factor), have recently been constructed. Preliminary attempts to add the genes for K100 capsular antigen have not been successful however. These hybrid strains will be compared to their rough parents for colonizing ability, serum sensitivity, and disease evoking capabilities.

Figure 1. IgA anti-X16 in intestinal loop secretions from animals with (open circles) and from animals lacking (closed circles) a Peyer's patch in their isolated loop. Standard error or mean indicated.



Project 3M762770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Work Unit 002 Pathogenesis of Enteric Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY						1. AGENCY ACCESSION <sup>3</sup>	2. DATE OF SUMMARY <sup>4</sup>	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SURMRY <b>76 10 01</b>	4. KIND OF SUMMARY <b>D. Change</b>	5. SUMMARY SCYT <sup>5</sup> <b>U</b>	6. WORK SECURITY <sup>6</sup> <b>U</b>	7. REGRADING <sup>7</sup> <b>NA</b>	8. DISCH'N INSTR'N <b>NL</b>	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT	
10 NO CODES*	PROGRAM ELEMENT <b>62770A</b>	PROJECT NUMBER <b>3M762770A802</b>		TASK AREA NUMBER <b>00</b>	WORK UNIT NUMBER <b>003</b>			
11. TITLE (Proceed with Security Classification Code) <b>(U) Histopathologic Manifestations of Military Diseases and Injuries</b>								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <b>002600 Biology</b>								
13. START DATE <b>63 08</b>		14. ESTIMATED COMPLETION DATE <b>Cont</b>		15. FUNDING AGENCY <b>DA</b>	16. PERFORMANCE METHOD <b>C. In-House</b>			
17. CONTRACT/GRANT		EXPIRATION:		18. RESOURCES ESTIMATE <b>PRECEDING</b>	19. PROFESSIONAL MAN YRS <b>FISCAL YEAR CURRENT</b>	20. FUNDS (In thousands) <b>318</b>		
21. DATES/EFFECTIVE: <b>08 08</b>		22. NUMBER* <b>NA</b>		23. AMOUNT: <b>78</b>	24. CUM. AMT. <b>5</b>	25. PERFORMING ORGANIZATION <b>Walter Reed Army Institute of Research</b>		
26. TYPE: <b>NA</b>		27. KIND OF AWARD: <b>NA</b>		28. PRINCIPAL INVESTIGATOR (Punish SBAAN II U.S. Academic Institution) <b>TAKEUCHI, AKIO, M.D.</b>				
29. RESPONSIBLE DOD ORGANIZATION <b>Walter Reed Army Institute of Research</b>		30. ADDRESS: <b>Washington, DC 20012</b>		31. TELEPHONE: 202-576-2024 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]				
32. GENERAL USE <b>Foreign intelligence not considered</b>		33. ASSOCIATE INVESTIGATORS <b>Hase, T., Ewing, E., Henley, G.</b>		34. NAME: <b>Inman, L.</b>				
35. KEYWORDS (Proceed EACH with Security Classification Code) <b>(U) Immune Responses; (U) E. coli; (U) Intestine; (U) Electron Microscopy; (U) Dysentery</b>								
36. TECHNICAL OBJECTIVE* <b>To define histopathologic manifestations of injuries and diseases which have current or potential problems in military personnel. The current effort is directed toward studies of enteric diseases and immunologic responses with infections. These studies provide a basis for a comprehension of pathogenesis, scientific treatment, and determination of prognosis in enteric infectious diseases of military personnel.</b>								
37. APPROACH, 38. PROGRESS (Punish individual paragraphs identified by number. Proceed with Security Classification Code) <b>Various morphologic techniques including histology, histo- and cytochemistry, autoradiography, immunofluorescent microscopy, transmission and scanning electron microscopy are employed. Various immunologic techniques have also been utilized.</b>								
39. (U) 76 10-77 09 In order to clarify the pathogenesis of diarrhea produced by E. coli in man, experimental enteric infections produced by peroral challenge with E. coli isolated from diarrheal stool, were studied by various morphologic techniques; the enteric lesions were characterized by mild entero-colitis with marked edema due to increased vascular permeability; at ultrastructural level the E. coli was capable of destroying the luminal aspects of intestinal epithelial cells including glycocalyx, microvilli and apical host cytoplasm and attached to the luminal plasma membrane of epithelial cells. Immunopathologic studies of the effect of oral rechallenge with virulent shigella upon parenterally and perorally immunized rabbits are in progress. Collaborative studies on rickettsial-host cells interaction in vitro have clarified the infectious cycles by electron microscopy; morphologic studies on mouse skin parasitized by larvae in relation to the transmission of rickettsiae demonstrated three definite types of stroma formation (see annual report, 76-77, Dept. of Hazardous Organisms, WRAIR).								

For technical report see Walter Reed Army Institute of Research Annual Progress Report,  
1 Jul 76-30 Sep 77.

\* Available to contractors upon originator's approval.

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Project 3M762770A802 MILITARY INTERNAL MEDICINE

Task 00 Military Internal Medicine

Work Unit 003 Histopathologic Manifestations of Military Diseases and Injuries

Investigators

Principal: Akio Takeuchi, M.D.

Associates: MAJ Edwin Ewing, MC; Tatsuo Hase, M.D.; SSG Garnett Henley, MS;  
E4 Lindsey Inman, BS; LTC Paul K. Hildebrandt, VC

Description:

To define histopathologic manifestations of injuries experimentally produced and diseases which present current or potential problems in military personnel. The current effort is directed toward studies of diseases of the digestive tract and immune responses due to infections and injuries. These studies provide a basis for a comprehension of pathogenesis, scientific treatment, and determination of prognosis in diseases and injuries in military personnel.

Approach to the Problem

A multi-disciplinary approach including conventional histology, histo- and cytochemistry, autoradiography, radio-tracer methods, various immunologic techniques, immunofluorescent microscopy, transmission and scanning electron microscopy is employed.

Progress

This work unit consists of studies of histopathologic manifestations of acute diarrheal diseases of infectious origin and collaborative studies of experimental rickettsial and trypanosomal infections with other departments of the WRAIR.

I. STUDIES OF HOST-PATHOGENIC MICROBE RELATIONSHIP IN THE DIGESTIVE TRACT

A. Studies on Histolysis and Ulceration of the Cecum  
Experimental in Entamoeba Histolytica Infections

Background

In man, acute diarrhea caused by E. histolytica is attributable to colonic lesions associated with invading amebae. The most common colonic lesions in human patients are acute ulceration which initially developed in the cecum. Cecal ulcers often complicate the prognosis of patients because they develop frequently into perforation of the bowel wall followed by peritonitis and also because they are an initial source of extra-enteric amebic lesions. Yet the pathogenesis of acute amebic ulceration has never been satisfactorily clarified. Some believe that ulcers develop from necrosis of bowel tissue by "lytic enzyme" produced by invading amebae, while others postulate that secondary invasion of bacteria is responsible for ulcer formation in the colon. This discrepancy had been mainly related to as yet unclarified mechanism of initial penetration of the gut mucosa by amebae and the subsequent early changes of the mucosal tissue surrounding invading amebae. Some believe that the ameba penetrated the epithelium by mechanical means. Others postulate that necrosis of gut mucosa by cytolytic enzymes produced by the ameba is responsible for penetration and establishing tissue infections.

By electron microscopy, Griffin (1972) and Pittman et al. (1973) studied rectal biopsy specimens from human patients with *E. histolytica* infections. Although these studies clarified several aspects of amebic-colonic mucosa interactions, they did not demonstrate penetration of colonic epithelium by amebae and their effect on the epithelial cell. This may be due to the limitations inherent in rectal biopsy material and the fact that human cases clinically encountered represent a relatively advanced stage of the disease.

It has been found that young germfree guinea pigs, inoculated intracecally with cultured *E. histolytica* and the enteric flora from a patient with acute amebic colitis, develop lesions similar to those of human amebiasis and thus provide a good experimental model for studies on the pathogenesis of amebic disease. Using this model at an early stage of infection when trophozoites invade the cecal mucosa, we have demonstrated for the first time, ultrastructurally, how amebae penetrate from the gut lumen to the lamina propria through the cecal epithelium and how cytoplasmic components of epithelial cells respond to penetrating amebae. (See Annual Report, 1973-74, Department of Experimental Pathology, WRAIR) and also early responses of mucosal cells and vasculatures to invasion of amebae (See Annual Report, 1974-75 and 1975-76, Department of Experimental Pathology, WRAIR).

We have continued to study these lesions. The present study is concerned with the subsequent changes of the cecum during tissue invasion by amebae. Our special attention is paid to analyze the pathogenesis of histolysis and ulceration of the cecum in this experimental amebae infection.

#### EXPERIMENTAL INFECTION AND MORPHOLOGIC METHODS

NIH Hartley strain germfree guinea pigs were used as experimental hosts. The animals were obtained by Caesarean section, maintained in Reynier's series 500 stainless steel isolators on dietary regimen L-445 and monitored at weekly intervals by procedures described (Phillips & Gorstein 1966). All animals were inoculated at the age of 12-17 days and each received a 1.0 ml inoculum containing 200,000 *E. histolytica*. CDC J-190 strain amebae were injected directly into the cecum during laparotomy under sodium pentothal anesthesia. They were maintained *in vitro* in Locke's egg-rice flour medium with enteric flora from the patient, incubated at 37°C and transferred thrice weekly. Inocula were prepared by pooling sediment from 48 hour cultures and quantitating with a hemocytometer. Control animals were treated just as the experimental group except they were inoculated with only the enteric flora without amebae.

Guinea pigs were killed at post-inoculation intervals of 7-12 days by ether anesthesia and autopsied in a conventional manner. The cecum was removed immediately and immersed in chilled physiological saline wherein the cecal wall was opened and the luminal contents carefully removed. Multiple sections were taken from mucosa of the cecum. Each section was divided into three pieces and processed for histochemistry (HC), light (LM) and electron microscopy (EM).

#### Results

Grossly the cecum of control animals appeared normal whereas the cecum of infected animals showed ulcers up to 2 cm in diameter, covered by gray or red-gray exudate. Adjacent to these ulcers, the cecal wall was generally edematous and hyperemic. Similar edematous and hyperemic areas were seen also independently of the ulcers. Elsewhere the mucosa appeared normal.

### Light Microscopy (LM)

The histologic appearance of the cecum of control guinea pigs was indistinguishable from that of conventionally raised guinea pigs of the same age (Takeuchi and Phillips, 1975; 1976). In contrast, the cecum of infected guinea pigs ranged from normal mucosa to histolyzed and ulcerated areas. In general the lysed areas of the cecum were characterized by the presence of dense amorphous material mixed with recognizable cellular debris, occasional erythrocytes and amebae. This amorphous material was occasionally overlying many of the surrounding congested and edematous areas. The lysed areas ranged from microscopic in size, scattered over the surface of the edematous and hemorrhagic mucosa to large ulcerated areas visible by gross observation. The former were found mainly in the epithelium, both at the surface (interglandular) and in the crypts (glandular), and in the immediate subepithelial portion of the lamina propria (Fig. 1). The large ulcerations involved the entire mucosa, extending to the muscularis mucosae, the Peyer's patches were occasionally involved.

Amebae were identified easily at the periphery of the lysed lamina propria and less frequently in the center of histolysis. Polymorphonuclear leukocytes (PMN) though abundant in the surrounding areas, could not be identified within the histolytic lesions. At the periphery of the lesions they were abundant in some cases and hardly recognizable in other.

The cecal mucosa and submucosa adjacent to histolytic lesions were thickened by marked edema, hemorrhage, PMN's, and congested blood vessels. The surface epithelium was thinned, with cuboidal cells showing shortened brush border and a pale cytoplasm (Fig. 1). The epithelial lining was infiltrated by PMN's. The crypts were elongated, tortuous, irregular in shape and often dilated when their lumen contained amebae (Fig. 1). The epithelium showed increased mitotic activity. Goblet cells, however, were decreased in number and often absent.

In some areas, a single ameba, or group of amebae, were in the process of penetration through the epithelium. In such instances, even when amebae were found in the lamina propria, the surrounding tissue often showed no evidence of histolysis. The lumen of capillaries and venules were engorged by red blood cells, PMN's, and aggregates of platelets. Extravasated red blood cells were commonly seen (Fig. 1), while amebae were rarely found within the vascular lumen. The histologic appearance of the mucosa away from the edematous and hyperemic mucosa was indistinguishable from that of control animals.

### Histochemistry (HC)

Enzymatic reactions in the brush border of the cecal mucosa of young guinea pigs are comparatively much weaker than in the small intestine (Jervis, 1965).

In infected animals enzymatic activity was completely lost in the lysed areas, generally decreased in the adjacent mucosa and was within normal range in the normal appearing mucosa. The outstanding exception was the overall increase in alkaline phosphatase reaction due to increased activity of elements in the lamina propria and especially to the influx of activated PMN's at areas adjacent to histolytic lesions. Lysed tissues and surface debris also showed intense, though more diffused, alkaline phosphatase activity.

The distribution of mucusubstances in epithelial elements of the normal cecal mucosa has been described in detail (Sheahan and Jervis, 1976). Neutral and sulfated mucin were found to be present in both brush border and goblet cells while

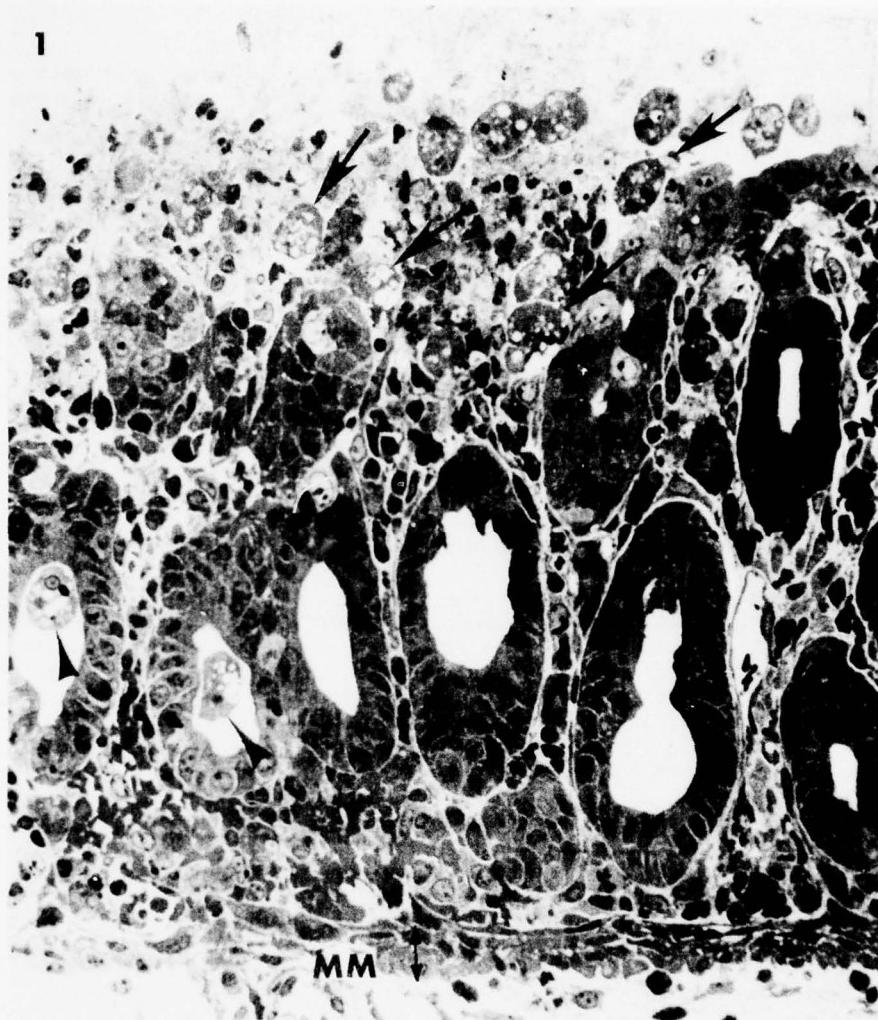


Fig. 1. Focal superficial histolysis with invasion of amebae in surrounding mucosa (arrows). Lytic tissue in the upper third of the mucosa shows dense amorphous material containing amebae and scanty cellular debris extruding into the gut lumen. The epithelium, on the surface at upper right and in the crypts, consists of low columnar cells without goblet cells. Crypt lumina contain amebae (arrowheads). PMN's are scanty but extravasated red cells are numerous, in particular, near the muscularis mucosae (MM). Epon section, methylene blue stain, X420.

sialomucin was only occasionally represented in goblet cells at the bottom of the crypts. Mucus discharge varied in the affected areas with complete disappearance of goblet cells in the vicinity of ulceration.

The normal cecal mucosa was found to contain no fats. In and around the areas of amebic invasion, however, fat was present, focally, in the cells of both surface and glandular epithelium and in the macrophages of the lamina propria.

#### Electron Microscopy (EM)

The ultrastructure of various elements of the cecal mucosa of control guinea pigs was indistinguishable from that of conventionally raised guinea pigs as previously described (Takeuchi and Phillips, 1975, 1976), as was the normal appearing mucosa in infected animals. In the lesions, EM observations confirmed those made by LM and clarified structural and histochemical changes. The center of histolytic areas, examined at low EM magnification, showed such an extensive degeneration of the host cellular components that the types of cell involved often could not be determined with any degree of certainty. At the periphery of lysed areas, however, the succession of changes in most cells could be readily identified (Figs. 2 and 3).

At higher magnification, the lysed areas consisted mainly of large amounts of fibrin, severely altered host cells and their cellular components. Most strands lacked striation. A few strands, however, showed distinct striations with typical periodicity fibrin ranging from 200 to 250 Å (Fig. 5). Two types of host cells could be recognized, namely, PMN's and epithelial cells. Both types were plentiful and in the process of severe alteration. Degenerating PMN's were smaller than their unaltered counterparts and showed homogeneous nucleoplasm and scanty cytoplasm without cytoplasmic granules (Fig. 4). Large numbers of desquamated epithelial cells were present mostly at the periphery of lysed regions and showed characteristic signs of degeneration such as intramitochondrial dense granules and cytoplasmic droplets (Fig. 3). Membrane-bound portions of host cytoplasm were numerous and generally lacked cytoplasmic organelles (Fig. 4). Mixed with these recognizable host structures were masses of nuclear material; some were enclosed by nuclear membranes, while others were in the process of nuclear lysis (Fig. 5).

Aggregates of membrane-bound dense granules were common; in favorable sections some granules appeared to be those of degenerating PMN's (Fig. 5) while others contained crystalline inclusions characteristic of eosinophilic leukocytes. Generally vascular structures could not be identified in areas of histolysis. In rare instances, however, severely altered capillaries and venules were recognizable and showed swollen endothelial cells with partial or complete lysis of cell membrane as well as occlusion of their lumen by fibrin deposits (Fig. 4). Clumps of bacteria were present in the histolysed areas but not elsewhere in the mucosa.

Adjacent to areas of histolysis, the mucosa, following invasion of amebae, showed various stages of change which have been described previously (Takeuchi and Phillips, 1976). Briefly, epithelial cells in the vicinity of invading amebae (Figs. 2 and 3) showed disrupted microvilli, dilated ER, altered mitochondria with dense matrix, characteristic dense granules, lipid droplets and deranged cristae (Fig. 3). PMN's exhibited condensation of nucleoplasm and cytoplasm, pinching off of granules containing cytoplasm, and cell membrane disruption with release of cellular content into the intercellular spaces. PMN granules when released extracellularly became much more dense and angular in shape. Mesenchymal cells, including macrophages, eosinophils, lymphocytes, smooth muscle

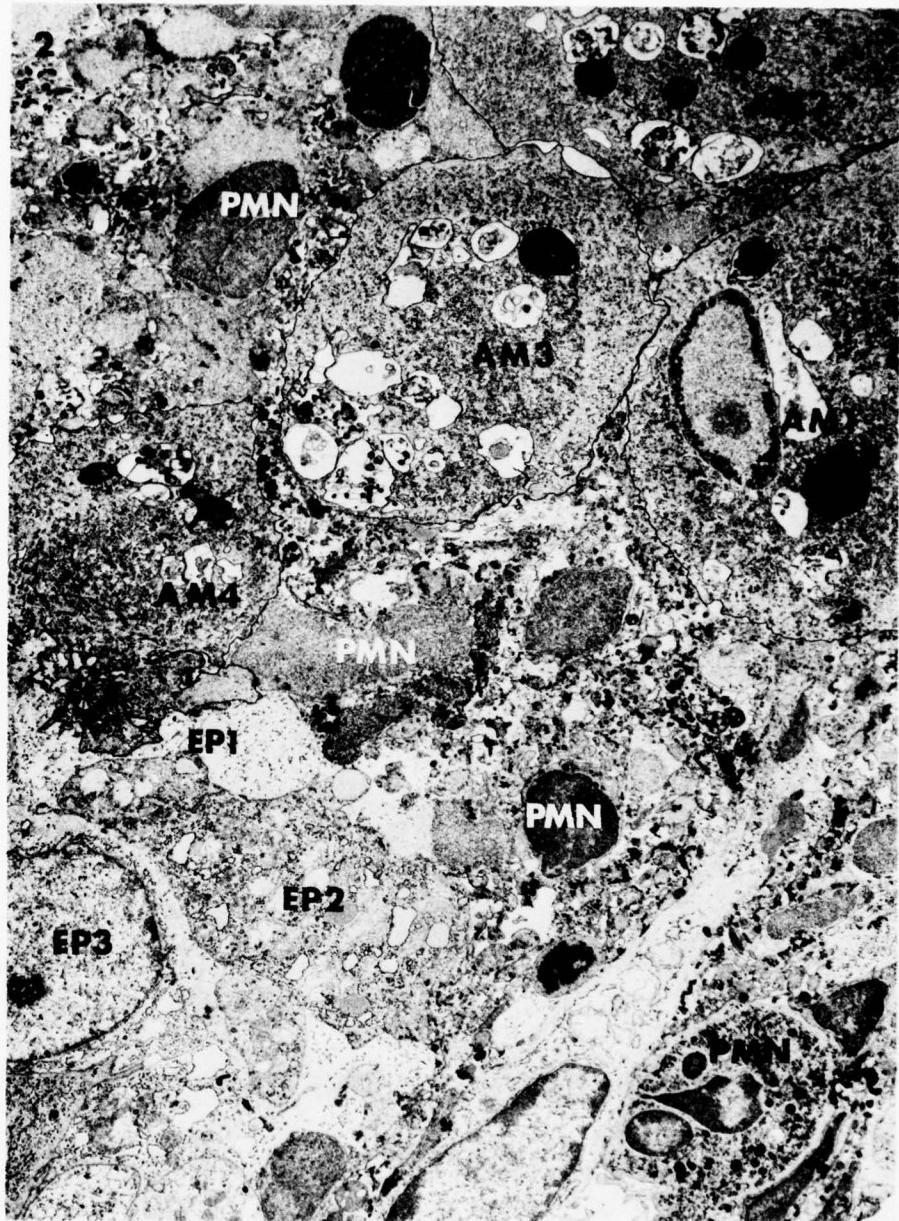


Fig. 2. Superficial mucosa at the margin of histolysis. Amebae (AM 1-4) are in the lamina propria near a crypt. Crypt epithelial cells (EP 1-3) in the proximity of amebae show cytoplasmic changes including swollen mitochondria, dilated ER and lipid droplets. The basal lamina of the crypt epithelium is not evident. PMN's near the surface (white letters) exhibit a variety of changes while PMN's deeper in the lamina propria (black letters) remain unaltered. Note extracellular dense granules around degenerated PMN's

X4,500.

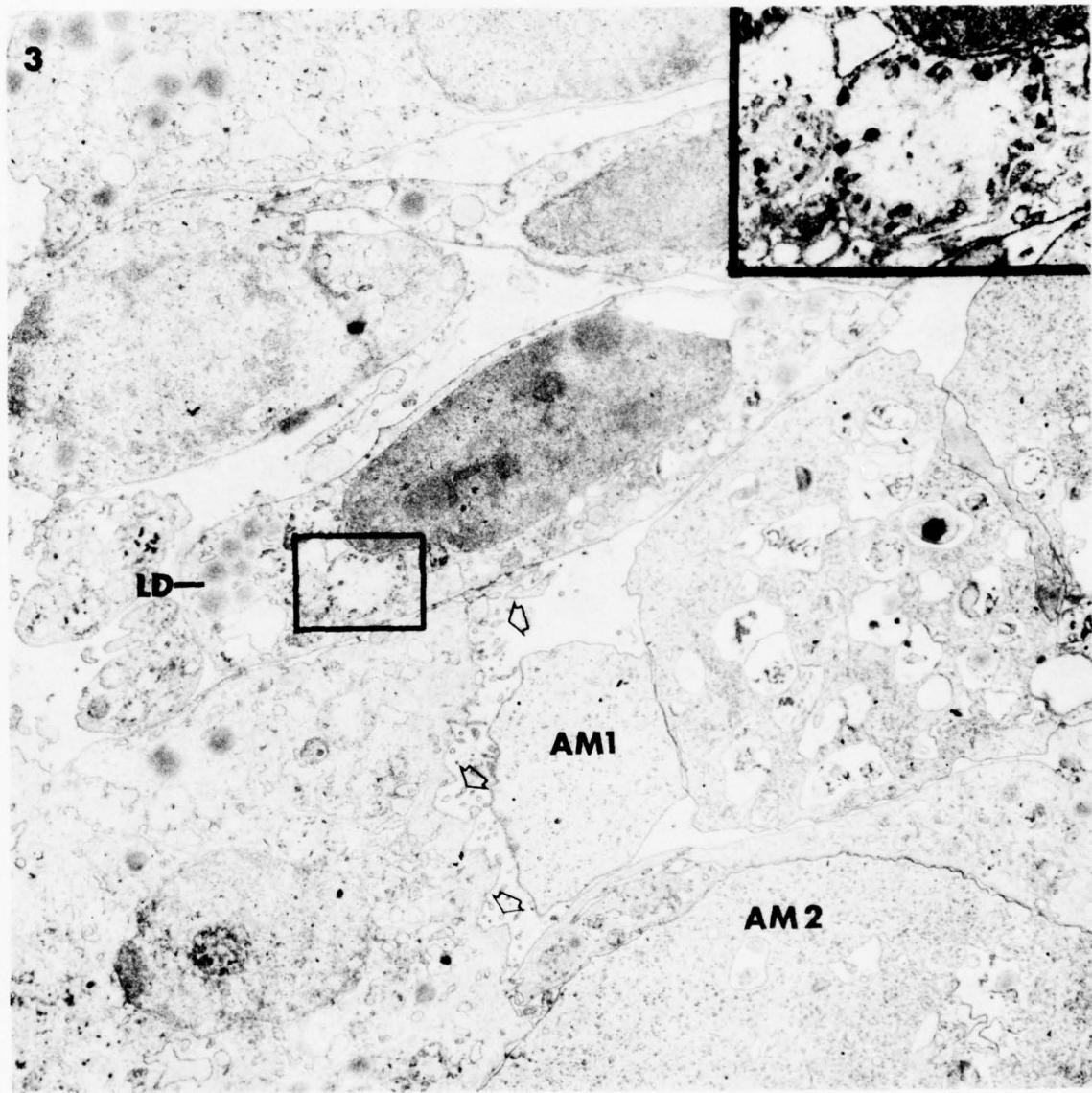


Fig. 3. Peripheral region of cecal mucosa adjacent to histolysis. Desquamated epithelial cells show severe degeneration of various components such as microvilli (arrow), mitochondria, ER. Lipid droplets (LD) are increased. Amebae (AM 1 and 2) are in contact with some of the epithelial cells. AM 1 may be a pseudopodium of AM 2. X6,000.

Inset enlarged from the square, exhibits swollen mitochondrion with altered cristae and characteristic dense granules. X14,000.

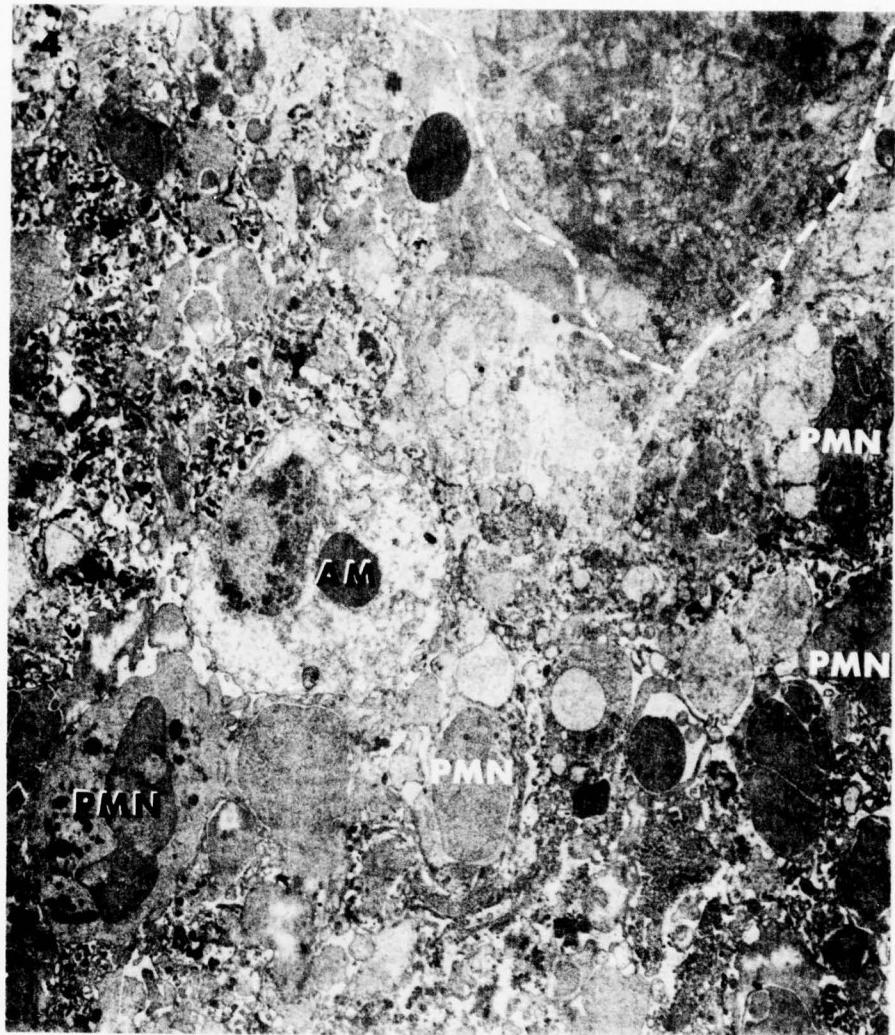


Fig. 4. Histolytic region of cecal mucosa. Near an amebae (AM), degenerating PMN's (white letters) showing characteristic segmented nuclei, and scant, dense cytoplasm, contrast with unaltered PMN (black letter). Note numerous membrane-bound portions of cytoplasm lacking cyto-organelles. In a capillary, outlined by white dashed line, endothelial cells are severely altered and the lumen is filled by fibrin. X4,500.



Fig. 5. Histolysis of cecal mucosa. Fibrin-like material is abundant; few strands show, at high magnification, characteristic striation of 220A (inset: X34,000) but most lack such striation. Extracellular nucleus is partly enclosed by nuclear membrane (arrows) which is in process of lysing. Cell membrane is absent in the remnant of a severely altered epithelial cell (EP). Note cytoplasmic portion of PMN's (PMN). X15,000.

cells and fibroblasts remained generally unaltered with only an occasional disruption of the cell membrane, when in close proximity to amebae and/or degenerating PMN's.

In the infected mucosa the majority of amebae appeared normal, only occasional amebae showed signs of severe degeneration such as homogeneous light cytoplasm with disrupted nuclear and cellular membranes.

#### Comments

Utilizing different morphologic techniques, this study has established that the cecum of our experimental model was characterized by distinct, severe, intestinal lesions represented by ulcerations and histolysis, surrounded by abnormal edematous and hyperemic mucosa which showed occasional tissue invasion by amebae. Areas of histolysis, even when microscopic in size, were always surrounded by the abnormal mucosa. Adjacent to these lesions, however, one could usually find areas of completely normal mucosa. Changes in the enzymatic activity of host cells in these lesions were clearly demonstrated by HC; these changes correlated well with EM findings. For example, the decrease of brush border and mitochondrial enzymatic activity paralleled closely the disruption of these cellular organelles. Likewise, increase in alkaline phosphatase activity correlated with the activation of the PMN's, while the diffuse activity of this enzyme in the lysed areas could be explained by the degranulation of these cells into the extracellular spaces.

Recent morphologic studies on human rectal biopsies with *E. histolytica* infection have shown that the most common colonic lesions, in addition to ulcerations, were nonspecific acute inflammatory lesions characterized by glandular hyperplasia, infiltration of neutrophils, stromal edema, depletion of goblet cells and hyperemia (Prathap and Gilman, 1970; Pittman, et al., 1973). According to Prathap and Gilman (1970), these lesions occurred in the complete absence of parasitic tissue invasion, while Pittman, et al. (1973) observed them with or without the presence of amebae. It is interesting to note that biopsy specimens containing no amebae were often erroneously diagnosed as ulcerative colitis or granulomatous colitis (Pittman, et al., 1973). In our experimental amebic infection, abnormal mucosa, similar to that described above, was consistently present adjacent to areas of ulceration and histolysis and was indistinguishable from that observed in the early mucosal and vascular responses to amebic invasion (Takeuchi and Phillips, 1976).

The amorphous material in histolytic lesions contained substantial amounts of fibrin. Masses of fibrin were also present both intraluminally and around the capillaries and venules in the adjacent abnormal mucosa. In addition, vasculatures showed a variety of conditions ranging from normal to necrosis of endothelial cells and thrombosis. The latter would presuppose severe ischemia and necrosis of the areas affected. It is also conceivable that the outpouring of plasma from inflamed and necrotic blood vessels into the extravascular spaces, stimulated the activation of clotting factors which undoubtedly led to rapid fibrin formation. On the other hand, one could expect that masses of extravascular fibrin could be easily lysed by the tissue fibrinolysin which is abundantly present in the gut (Myre-Jensen, et al., 1973). Indeed, in our model only a few fibrin strands showed typical striation, while most lacked such striations. This would indicate that the fibrin, regardless of its location, was in a state of partial lysis or polymerization.

One of the most puzzling aspects of amebiasis in both man and animals is leukotaxis in acute amebic lesions which show, in whatever organ they are found, necrosis and clumps of PMN's. Leukotaxis coexists with a lack of acute reaction

in nearby areas even in the presence of clusters of invading parasites. Thus Brandt and Tamayo (1970) have questioned that amebae are truly leukotactic. Support for such an hypothesis is provided by the differences observed at the LM level between stool smears of two different types of dysentery in man, namely, bacillary and amebic dysentera. In the former, the stool is characterized by the presence of numerous unaltered PMN's mixed with erythrocytes and cellular debris, a truly cellular exudate; while in the latter, the stool shows a complete or almost complete absence of recognizable PMN's (Stamm, 1970). These observations are in agreement with those made in this and in a study of bacillary dysentery (Takeuchi, Jervis, and Formal, 1975).

Recent histologic (Prathap and Graham, 1970; Pittman, et al., 1973) and EM (Griffin, 1972; Pittman, et al., 1973) studies of rectal biopsies of human amebiasis indicate that PMN's are abundant in the colonic mucosa. The present study confirmed the above observations and provided additional findings on migrating PMN's which were well documented by the characteristic alkaline phosphatase reaction and EM. They were indeed plentiful even in areas of histolysis where lysed PMN's discharged their granules into the extracellular spaces. This massive disintegration of PMN's appears to be peculiar to amebic infections and would be responsible for their absence in the stool.

It has been shown that in an immune-induced acute inflammation of the gut, the immune complex in the gut lumen attracts PMN's from the lamina propria (Bellamy and Nelson, 1974). A similar chemotactic mechanism may be operative in enteric amebiasis. No attempt, however, was made during this study to explore such a possibility.

Usually PMN's serve a defensive role, through phagocytosis of cell debris and infectious microbes. In our present and previous studies (Takeuchi and Phillips, 1975, 1976), PMN's do not seem to affect the amebae but degenerate rapidly under the influence of this parasite. Furthermore, they appear to act as a potentially injurious agent to tissue elements at sites of acute inflammation. PMN's, indeed, have been found to be one of the contributing factors to tissue damage in a wide variety of inflammatory conditions induced by many challenging agents (Cochrane and Janoff, 1974). The role of PMN's as mediators of tissue injury is well documented by their involvement in the Arthus reaction (Udaka, 1971) and in the local Shwartzman reaction (Taichman, 1971). In fact, some details of the extracellular release of PMN's observed in this study, are indistinguishable from Figures 5 and 6 illustrated in the work of Taichman (1971) on the local Shwartzman reaction, while other details are similar to the mechanism of extrusion of PMN lysosomal contents in the presence of an immune complex (Cochrane and Janoff, 1974). However, it is also possible that the massive release of PMN granules observed in our studies may well be a nonspecific reaction.

Whatever the precise interrelationship or mediatory mechanism might be, the present observations strongly suggest that, in addition to tissue ischemia related to vascular thrombosis and necrosis, PMN's may play a major role in the characteristic histolytic process of the gut mucosa in acute amebic colitis.

#### Recommendation

Further studies on the role of PMN leukocytes should clarify the precise pathogenesis of enteric lesions produced by *E. histolytica*. For this purpose, we are planning to study interactions between PMN leukocytes and intestinal mucosal tissue in organ culture and also to study early colonic lesions of human patients with *E. histolytica* infection by rectal biopsies.

## B. STUDIES OF INTESTINAL INFECTIONS WITH PATHOGENIC E. COLI

### Background

Some strains of Escherichia coli (E. coli) are capable of producing diarrhea in various animal species, including man (Formal, et al., 1973). It is generally accepted that there are two mechanisms by which E. coli can cause diarrhea. The enterotoxigenic or noninvasive strains secrete heat stable or heat labile enterotoxins which are responsible for the diarrhea. In addition to the production of the enterotoxins several members of this group have been shown to attach to the surface of the gut epithelium. The invasive E. coli, on the other hand, penetrate the enteric epithelial barrier and produce an enteritis.

Recently Cantey and Blake have isolated a strain of E. coli 015 (RDEC-1) from rabbits with diarrhea and found that this strain lacked both invasiveness as tested by HeLa cell and Sereny tests and enterotoxigenic activities determined by rabbit ileal loop.

Young rabbits when challenged orally with the above strain, developed diarrhea. We clarified morphologic features of experimental intestinal infections of rabbits inoculated orally with strain RDEC-1 of E. coli 015, as observed by FA, light microscopy (LM), transmission (TEM) and scanning electron microscopy (SEM).

### Materials and Methods

Bacterial Culture. Inoculum of South Carolina strain E. coli 015 RDEC-1 were transferred to pen-assay broth and cultured at 37°C for 18 hours. The details of the challenge culture technique have been previously described elsewhere (Cantey and Blake, 1977).

Animals. Healthy, young New Zealand white rabbits (Walter Reed strain) of both sexes, weighing 1.7-1.0 Kg. were used.

Infection. Animals were infected with 8 ml. of broth containing 10<sup>9</sup> organisms administered by stomach tube. Control animals received 8 ml. of sterile broth. The details of the method have been described previously (Cantey and Blake, 1977). At seven days after oral challenge, infected animals usually developed watery diarrhea which reached its peak at about ten days when they were sacrificed by injection of a lethal dose of sodium pentobarbital.

Specimen sampling. Segments of ileum and cecum from both infected and control animals were removed, cut and fixed into five pieces and processed as follows:

FA. The first pieces were frozen in isopentane in dry ice, sectioned and stained with specifically labeled fluorescent antibody to RDEC-1 and counterstained with rhodamine-conjugated bovine serum albumin (Cantey and Blake, 1977).

LM. Second pieces were fixed in formalin and divided into two portions. A portion of each of the formalin fixed tissues were embedded in paraffin. Individual sections of paraffin embedded material were stained either with hematoxylin eosin or Giemsa.

Thick sections from Epon embedded tissues were stained with Toluidine-Blue-  
Pyronin Y.

SEM. The remainder of the formalin fixed tissues were post-fixed in Osmium, washed then dehydrated. Tissue was then placed in amyl acetate dried in a critical point apparatus, mounted, coated with Gold Palladium and examined by SEM (Takeuchi and Zeller, 1972).

TEM. The remaining 3 pieces were cut and further processed according to one of the following three procedures: 1. routine glutaraldehyde fixation; 2. Alcian blue-Lanthanum fixation (AB-L) according to the method of Shea (1971); Ruthenium red fixation (RR) by Luft (1971).

Tissues in the above three different fixations were processed according to our routine EM methods.

#### Observations

FA. Large numbers of fluorescent bacteria were present in the lumen of the ileum, cecum and colon. The ileal, cecal and colonic epithelia were layered with large numbers of RDEC-1. Uninterrupted involvement of the epithelium were most often seen in the ileum, cecum and colon and least often in the jejunum. Fluorescent bacteria were occasionally seen in the smears of luminal contents of the jejunum but were not seen adhering to the jejunal epithelium. The bacteria seemed to adhere to the epithelium in a layer one bacterium thick. E. coli RDEC-1 were not seen in the lamina propria.

LM. Paraffin sections of the ileum and cecum showed a considerable spectrum of structural changes representative of a moderate acute enterocolitis, characterized by extensive edema involving the lamina propria and extending, beyond the muscularis mucosae, into the submucosa. The muscular coats, however were not edematous. A small number of polymorphonuclear (PMN) leukocytes were consistently present in the lamina propria. The blood vessels were congested but no hemorrhage was observed. The most striking feature was seen at the luminal border of the epithelial cells of both the ileum and cecum which, in HE stained sections, appeared coated by a thick basophilic layer, irregularly hazy. This layer was present at the surface of the villi in the ileum and on the surface of the cecum (Fig. 6), including the crypt openings. In contrast, the brush border of the control ileum and cecum was thin, regular and eosinophilic. In favorable sections, stained with Giemsa and examined at high magnification, the basophilic layer was seen to consist of individual bacilli, aggregated along the brush border region. The epithelium was generally columnar and most often cuboidal, the degree of change being roughly proportional to the severity of the infestation. The goblet cells were decreased in number and, in areas of heavy infestation, often absent. The relationship between epithelium and bacterial was seen more clearly in thick sections of Epon-embedded tissues (Fig. 7). The luminal surface was ragged; the infested epithelial cells generally lost their normal columnar shape and occasionally contained pale cytoplasm and cytoplasmic vacuoles. In isolated areas, these infected epithelial cells appeared to pile upon each other and seemed to be in the process of shedding from the epithelial lining into the gut lumen (Fig. 7).

SEM. The surface of the ileal mucosa of control rabbits was similar to that of the normal rat (Carr and Toner, 1968), while the mucosal surface of the control cecum and colon bore a close resemblance to that of the normal monkey (Takeuchi and Zeller, 1972).

At magnification up to 300 times, the mucosal surface of the ileum, cecum, and colon infected with RDEC-1 was indistinguishable from that of control rabbits.

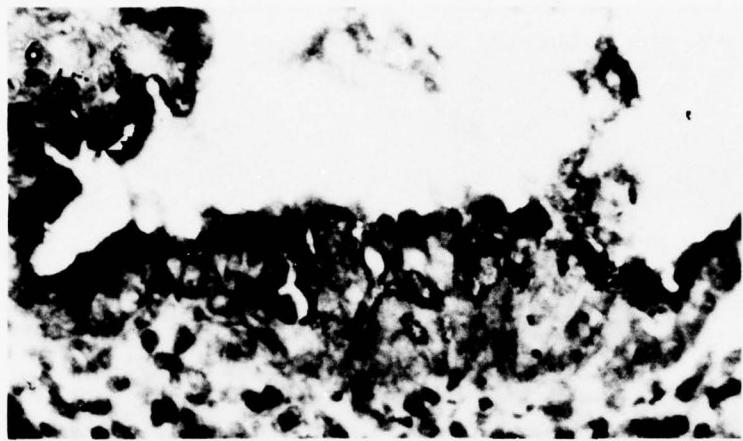


Fig. 6. Oblique section of infected cecum. MVB of the surface epithelium is uniformly covered by heavily stained material which represents numerous RDEC-1, while such stained material is absent on the crypt epithelial surface. Goblet cells are decreased in number. A small number of PMN leukocytes are seen in the edematous lamina propria. Paraffin section stained with Giemsa; X360.



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Fig. 7. Infected cecal epithelium, showing multiple *E. coli* at the brush border. The cytoplasm of many infected epithelial cells is pale while others show unaltered cytoplasm. A mass of infected epithelial cells in the center project into the gut lumen and appear to be in the process of shedding. Arrows indicate the basal lamina of the epithelium. Epon section, Toluidine blue-Pyronin Y stain; X1,000.



Fig. 8. SEM view, surface of the ileal mucosa of infected rabbit with RDEC-1. Bacilli are aggregated and piled on each other. Elongated microvilli are present adjacent to the aggregates of bacteria (arrows). The surrounding epithelial surface appears unaltered.  
X6,600.



Fig. 9. SEM view, aggregates of bacilli on the mucosal surface of the cecum. Individual bacilli are in contact with each other and are attached to the epithelial surface. The interface between bacteria and MVB is not clear. Note elongated microvilli (MV) adjacent to bacterial attachment. X10,700.

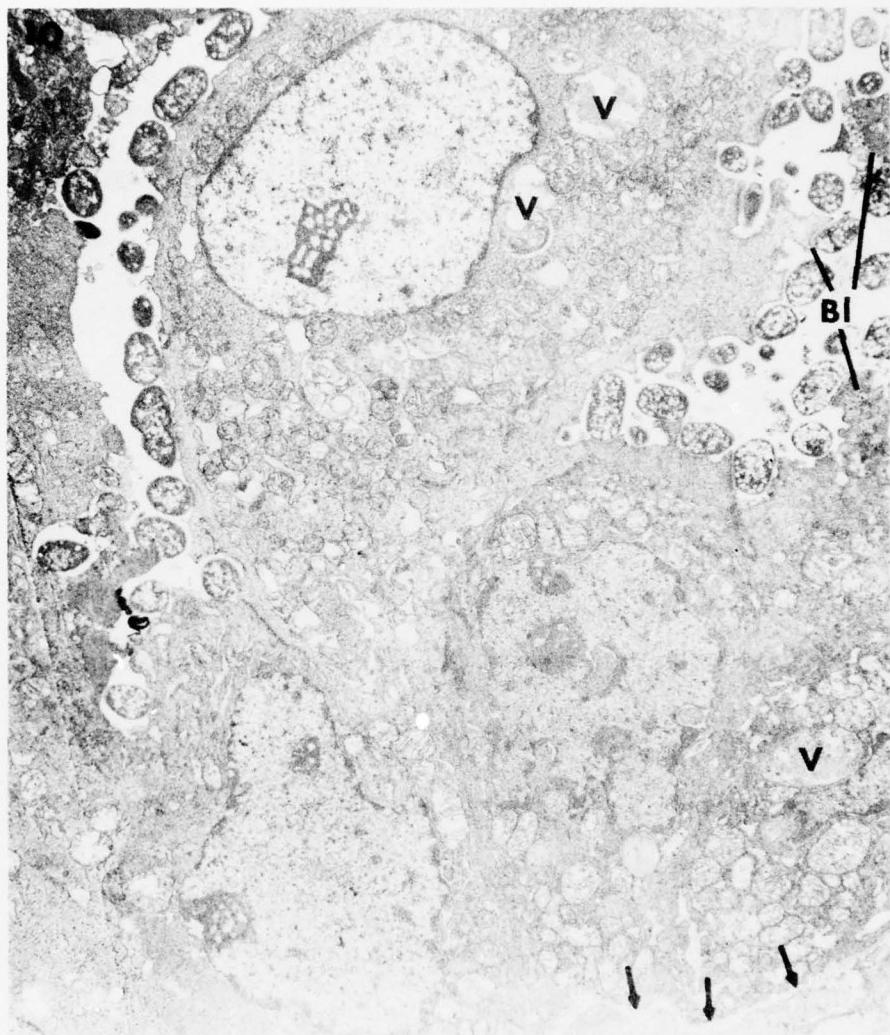


Fig. 10. Infected cecal epithelial cells showing multiple bacteria at the cell surface. Glycocalyx and microvilli are totally absent. Infected epithelial cells are low columnar and contain increased number of vesicles and vacuoles (V), in their cytoplasm. Apical cytoplasm proximal to bacterial coherence shows cytoplasmic invaginations, projections, and blebs (BI). Arrows indicate the basal lamina of the epithelium. The lamina propria contains finely granular material indicating intensive edema. AB-L, lead citrate-uranyl acetate stain; X6,500.



Fig. 11. Bacteria are closely abutted to the host trilamellar membrane. The glycocalyx and the microvilli are totally absent in this routinely fixed tissue. Note that a number of slightly electron dense and relatively well-defined structures are identified around bacterial cell wall. Cw = Cell wall; Hm - Host cell membrane. Routine fixation without cytochemical treatment, lead citrate-uranyl acetate stain; X51,000.



Fig. 12. Bacteria are closely approximated to the epithelial cell membrane (Hm). Note that remnant of Lanthanum stained material is still present on the host cell surface (L) and also between the bacterial cell wall (Cw) and host membrane. AB-L treatment, lead citrate-uranyl acetate stain; X57,160.

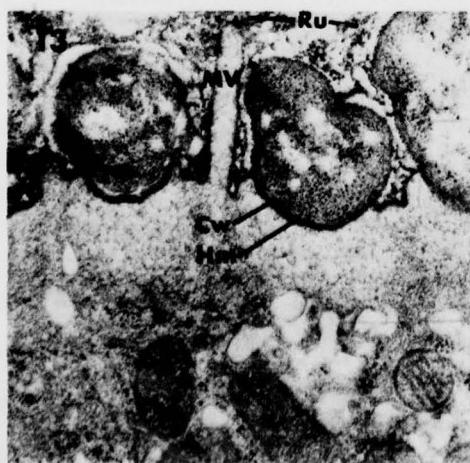


Fig. 13. Bacteria are intimately associated with host cell membranes (Hm) and an epithelial cell. Remnants of microvilli (MV) are covered by ruthenium red positive glycocalyx (Ru). RR treatment, lead citrate-uranyl acetate stain; X34,000

At magnification ranging from 300 to 1000 times, aggregates of bacteria were found on the mucosal surface of infected ileum and large intestine; their distribution was in a patchy fashion in the ileum and colon and was generally diffuse in the cecum. However, the mucosal surface between bacterial aggregates was not remarkable. In contrast the control intestines were free of bacterial aggregates on the mucosal surface.

At magnification ranging from 1000 to 5000 times, individual bacilli were clearly discernible in the areas of bacterial aggregates and appeared to contact each other and attach closely to the MVB (Figs. 8 and 9). The exact interface between bacteria and MVB could not be resolved with certainty. In the proximity of the bacterial attachment, microvilli were elongated and projected into the lumen (Fig. 8), whereas, the surface distal to the bacterial attachment showed the individual tips of unaltered microvilli.

TEM. Ultrastructural observations confirmed IM findings and revealed further details. Although the bacterial infestation at the brush border of the epithelial cells was much more heavy and diffuse in the cecum than in the ileum, *E. coli* epithelial cell interactions were essentially the same in both. The following TEM observations therefore, were made on the cecum. Under routine glutar-aldehyde fixation, in the rabbit cecum, the glycocalyx was seen only occasionally as an electron-opaque, fine material which radiated from the tip of the microvilli. In contrast, after RR treatment the glycocalyx was easily identified as a layer of evenly distributed dense material up to 500 nm thick, whereas AB-L stained it as a layer of moderately dense and coarsely granular material, approximately 300 nm thick, which was sparsely distributed over and around the microvilli.

In the infected cecum, examined at low magnification, innumerable bacteria were seen near or on the luminal surface of the columnar epithelial cells but not on the goblet cells when these were present. Bacteria were arranged randomly, side by side, on the cell surface, seemingly attached to the plasma membrane either parallel or perpendicular (Figs. 10-13) to it. Some of the epithelial cells involved seemed in process of desquamating into the lumen.

At higher magnification, bacteria were individually identified; they measured up to 4  $\mu\text{m}$  in length and 1.5  $\mu\text{m}$  in diameter, and exhibited the characteristic structure of gram-negative bacterial (Figs. 11-13) with occasional binary fission. Electron opaque, well defined spherical structures, reminiscent of bacterial pili, were occasionally identified at or around the bacterial cell wall (Fig. 11). Both the RR and the AB-L techniques showed, that in close proximity of bacteria the glycocalyx was completely lost as were the microvilli and the terminal web. In addition, in the more severely affected epithelial cells, the apical cytoplasm also was destroyed and replaced by numerous bacteria. In areas not adjacent to the bacteria, few blunt microvilli remained on the irregular cell surface; they were still covered by glycocalyx (Fig. 13). The cell wall of the bacteria, where it closely abutted the cell membrane of the host cell, was separated from it by an 11 nm space. The latter showed invaginations, projections, and blebs (Fig. 10) with the invaginations containing at times pili-like structures (Fig. 11). Some of the projections and blebs, together with coherent bacteria, appeared to be shedding from the host cytoplasm into the lumen. When bacteria were found at the intercellular tight junction, the junctional complex was displaced, without altering, however, the structural integrity of the complex.

In infected cells, cytoplasmic organelles showed a variety of changes. Cisternae of rough and smooth endoplasmic reticulum as well as the Golgi apparatus were often dilated; vesicles and vacuoles of different size were easily identified (Fig. 10). The mitochondria were swollen and showed deranged cristae in a light, granular matrix. Membrane-bound lipid droplets were common. There was also an increased number of phagosomes which contained altered host cytoplasmic components. The nuclei, however, remained unchanged. In rare instances, bacteria were identified in the perinuclear region where they were either enclosed in membrane-bound vesicles or vacuoles, or more rarely, free in the cytoplasm, but never in the lamina propria.

The majority of the noninfested epithelial cells remained unchanged, although some of them, either topographically close to or away from bacteria showed shortened, blunt microvilli, occasional phagosomes and an increased number of vesicles.

#### Comments

The initial description of RDEC-1 diarrhea, which was based on FA and standard histological techniques for IM (Cantey and Blake, 1977) revealed: a) Great numbers of RDEC-1 *E. coli* localized at and associated with the luminal surface of the villus and surface epithelium of the small and large bowel, and b) that the MVB and part of the epithelial cell cytoplasm were destroyed in the presence of the attached bacteria.

In the present EM observations, SEM has shown how RDEC-1 *E. coli* inhabit their natural environment; three dimensional views revealed how bacteria intimately populated the surface of the gut mucosa. TEM has clearly demonstrated that RDEC-1 *E. coli* multiply, destroy the MVB and closely abut the luminal plasma membrane of epithelial cells.

The ability to adhere to mucosal surfaces which, in some cases, may be mediated by bacterial pili, is thought to be important for bacteria that infect such surfaces (Smith, 1977). Those who would apply these two allied concepts to the area of bacterial diarrhea have based their opinions on data obtained in highly artificial systems utilizing separated gut epithelial cells, intestinal epithelial strips; isolated MVB, neonatal animals, and the rabbit ileal loop (Freter, 1973; Jones and Rutter, 1972; Moon, et al., 1977). All such studies have failed to consider that IM cannot resolve the fine details of the relationship between bacteria and the MVB of the epithelial cell surface or that several barriers stand between bacteria and the MVB in the intact animal, including the glycocalyx (Ito, 1969), the mucus layer, and the unstirred water layer (Dietsch, 1975). Evidence against adherence of bacteria to MVB was obtained in the present study. RDEC-1 was found adhering not to the MVB but to the surface of mucosal epithelial cells that had lost their MVB. Although we were not able to demonstrate with certainty that the pili were responsible for, or involved in the bacterial-host cell interaction, some sections did reveal pilus-like structures along the bacterial surface (Fig. 8) and within the cytoplasmic invaginations in the area of contact between the bacteria and the host cell membrane. The narrow 11 nm space between the bacteria and the host cell membrane would, however, appear to mitigate against the possibility that these structures are pili. The space could easily contain RDEC-1 capsular material that stains well with ruthenium red.

TEM studies in enteric infections with *Salmonella typhimurium* in guinea pigs (Takeuchi, 1967), *Shigella flexneri* in guinea pigs (Takeuchi, et al., 1965, and monkeys (Takeuchi, Sprinz and Formal, 1968), and invasive *E. coli* in rabbits have revealed that the bacteria passed through the gut epithelial barrier, destroyed

the MVB in the region of bacterial penetration, and produced a severe inflammation in the lamina propria. On the other hand, it has been shown at the fine structural level that several microbes characteristically destroy the MVB of the gut epithelial cells and attach preferentially to the luminal plasma membrane of host cells. They include spirochetes and flagellated microbes on the mucosal epithelium of the colon of monkeys and man (Takeuchi, et al., 1974), segmented, filamentous microbes in murine ileum (Davis, et al., 1974) and Cryptosporidia in the lumen of guinea pigs (Vettering, et al., 1971). These microbes, however, cause no acute inflammatory response in the gut mucosa. It is evident that injury to the microvillous membrane region and the attachment of microbes to the host membrane does not necessarily cause acute inflammation. Yet, other studies have provided evidence that some strains of *E. coli* can penetrate the glycocalyx and contact the MVB with pilus-like appendages, but without causing damage to the MVB. RDEC-1, in contrast, is able to: 1) destroy the microvillous membrane and the apical cytoplasm of mucosal epithelial cells, 2) produce a mild to moderate acute inflammatory response in the lamina propria, and 3) elicit a diffuse and intense edema in the lamina propria. The destructive and inflammatory capabilities of RDEC-1 may be due to the small amount of *Shigella dysenteriae*-like enterotoxin that it synthesizes (O'Brien, et al., 1977). The edema may be due to the release of vasoactive amines such as serotonin and histamine, both of which are abundant in the intestinal mucosa.

Detailed ultrastructural observations of RDEC-1 *E. coli* diarrhea in the rabbit have thus shed new light and raised additional questions concerning the understanding of bacterial-host cell interactions and mechanisms of *E. coli* diarrhea. They did not resolve the question of whether pili and/or capsular material are responsible for bacterial adherence to epithelial cells.

#### Conclusion and Recommendation

In RDEC-1 *E. coli* infection in experimental animals, the LM, FA, SEM and TEM studies provided new information on pathogenic *E. coli* diarrhea. We have initiated immunopathologic studies on the effect of rechallenge with RDEC-1 upon systemic and peroral immunized animals with killed and live RDEC-1.

## II. COLLABORATIVE STUDIES ON TRYPANOSOMA AND RICKETTSIAL INFECTIONS

### A. Trypanosoma Infection

#### Background

The fine structure of *Trypanosoma brucei* has been studied by a number of investigators (Fuge, 1969; Vickerman, 1969; Macadam and Herbert, 1970; Wright and Hales, 1970; Hecker, et al., 1972; Steiger, 1971; Smith, et al., 1974; Bohringer and Hecker, 1975; Hogan and Patton, 1976). Although these studies have provided important information on the fine structure of this organism, we still lack much knowledge regarding the specific structure of trypanosomes, the flagellar pocket (FP).

Utilizing freeze-etching (FE) and conventional thin-sectioning techniques (TS) of electron microscopy, the present study describes the morphology of the apical part of the FP and attempts to relate the EM observations to the dynamic function of FP.

#### Results and Comments

The results presented here were accumulated from observations of replicas of freeze-cleaved pellets and thin-sectioned images of long slender bloodstream forms of *T. brucei*.

Cross sections through the open apical FP showed double tubular bundles which extend longitudinally through the flagella. The paired tubules in each bundle were partially separated by a radian that projected outward toward the flagellar sheath (FS). When open, the apical FP showed an enormous amount of space between the FS and the FP membrane (Fig. 14).

The FP of longitudinally-sectioned *T. brucei* was a bulb-shaped depression in the body, continuous with the pellicle and FS (Figs. 15-17). There were opposing sides of the FP in sections cut along this plane; one side was adjacent to the body, while the other was flanged (approximately  $1 \mu$  long) anterior to the basal granule (Figs. 15 and 16). The apical part of the pocket on the flanged side compressed the FS forming part of a "neck region (NR)" (Figs. 15-17). This region was approximately  $0.5 \mu$  long and extended into the FP. The NR was completed on the side opposite the flagella where the body contacted the FS (Figs. 15-17). The flanged side of the FP, in longitudinally sectioned cells, was populated by electron opaque material (Figs. 15 and 16). Microtubule-like striations of the pellicle were seen in sections cut oblique to the cell's longitudinal axis (Fig. 17). Flocculent material was entrapped in pinocytotic vascuoles beneath the FP (Fig. 16).

The FP appeared closed when fractured along favorable planes in freeze-cleaved cells (Figs. 18 and 19). The flanged part of the FP closely apposed the FS so that no discernible space was seen between them (Fig. 18). At higher magnifications, the FS on one side was convoluted with contact creases and rolls that insulated the FP from the environ (Fig. 19).

*T. brucei*'s FP membrane is without cytoskeletal structures, thereby possibly rendering it the most flexible part of the pellicle (Langreth and Balber, 1975). The absence of microtubules under the pocket membrane could have evolved through the constant diffusion of FP media into the cytoplasm, and vice versa. Membrane uptake during pinocytosis (a phenomenon common to the plasmalemma of some free-living ameba, Chapman-Andresen, 1971) could also have prompted the exit of micro-

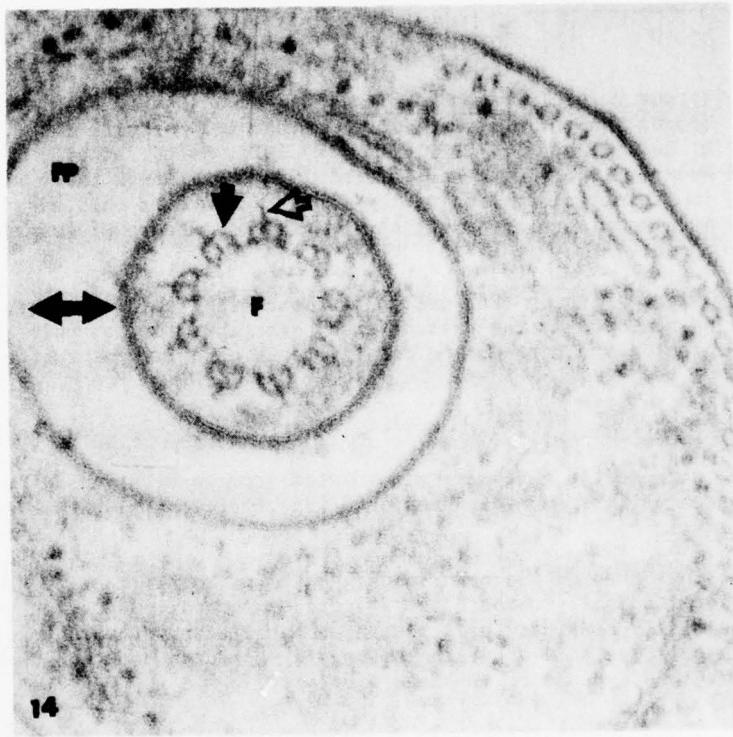
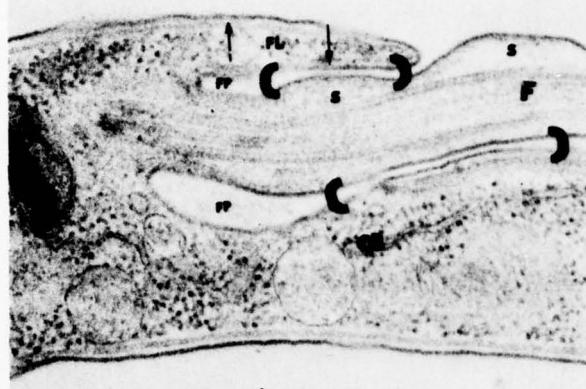
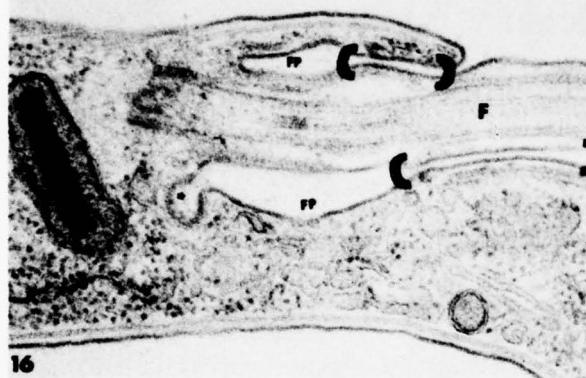


Fig. 14. Cross section through the apical flagellar pocket (neck region). The flagella in this region has double tubular bundles (closed faced arrows) each of which is separated by a radium (open faced arrows). When open, the apical flagellar pocket has space between the flagellar sheath and FP membrane (double arrow head). X81,000.



15

Fig. 15. Longitudinally thin-sectioned *T. brucei*. The flagellar pocket (FP) is a flask-shaped depression into the body of the cell, continuous with the pellicle (arrows) and flagellar sheath (S). Surrounding the FP is a body flange (FL) dotted by compact material and the body of the cell (CB). The (S) is at times embraced by the FL and CB forming a neck region (parenthesis) at the mouth of the FP. X49,000.



16

Fig. 16. Longitudinally thin-sectioned *T. brucei*. Sealing of the FP at the neck region compacts entrapped flocculent material into pinocytotic vacuoles (asterisk). X49,000.

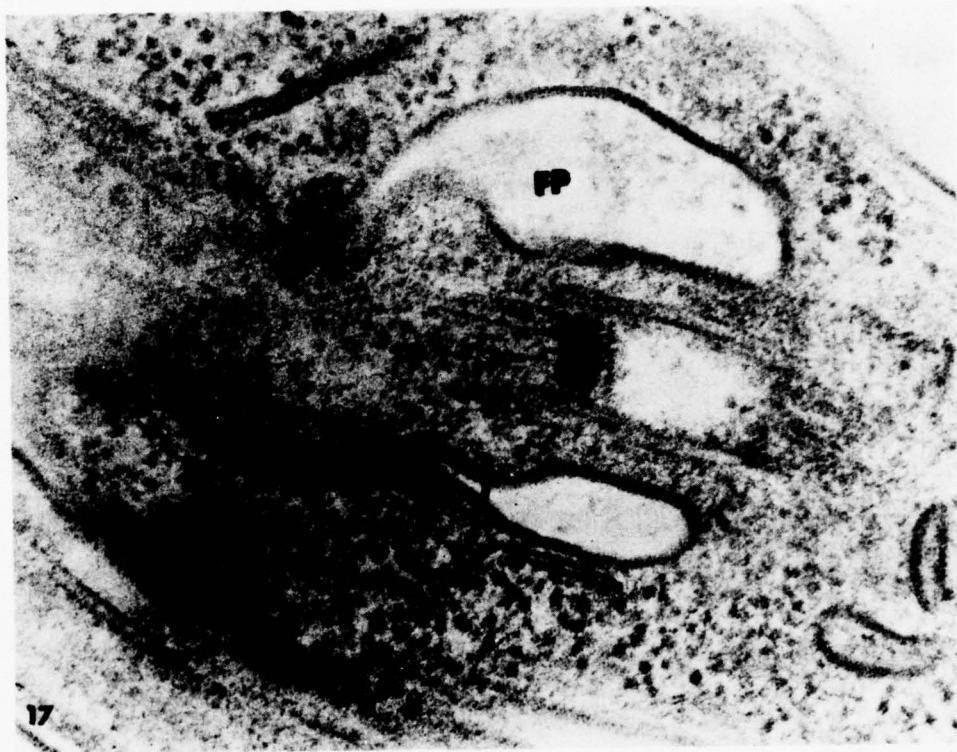
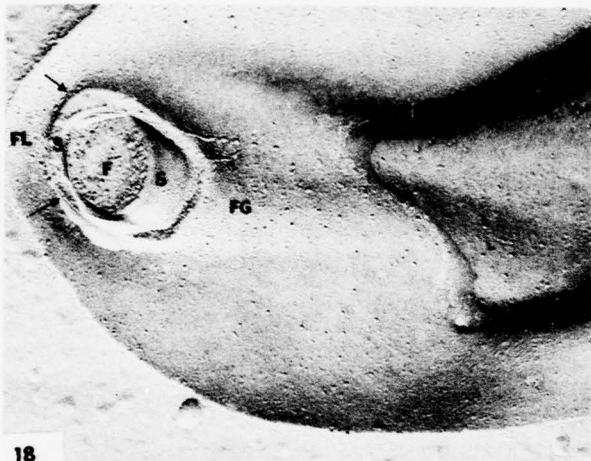
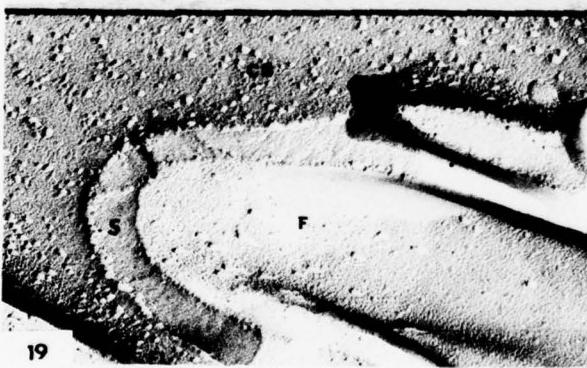


Fig. 17. Oblique thin-sectioned T. brucei. Membranes in the neck region (parenthesis) are reinforced by microtubule-like striations of the pellicle. X61,800.



18



19

Fig. 18. Freeze-fractured neck region. The flagella (F) is cleaved at the surface along the flagellar groove (FG). The flanged side of the body (FL) has embraced the flagellar sheath (S), sealing the flagellar pocket from the environ (arrows). X49,000.

Fig. 19. Freeze-fractured neck region. Contact of the flagellar sheath (S) by the body of the cell (CB) and flanged side (FL) causes rolls and creases in the flagellar sheath (S). X77,000.

tubules from the FP membrane. Pinocytosis in the FP occurs juxta the base of the flagella (Brown, et al., 1965), and not in the area of the NR.

Microtubule-like striations of the FP membrane just inside the NR, as described in this study, suggest a possible reinforcement of that membrane as shown in sections cut slightly oblique to the cells longitudinal axis.

Evaluation of results obtained during this study has led us to believe that the NR greatly influences pinocytosis and the uptake of microparticles through the FP. Movements of the flagella and undulating membrane, as well as contortions of the body during locomotion probably cause the NR to open and close, allowing media and microparticles to enter and exit the FP. Pressure created by the current of media into and out of the FP would then cause the FP to expand and contract. Expansions of the FP would draw media into the FP and probably close the NR; contractions would expel fluids into the environ, thereby opening the FP at the NR. Closing the pocket would allow heavier particles to settle on the FP membranes and later to be washed deeper into the pocket where they then compact into pinocytotic vacuoles near the base of the flagella.

#### B. Studies on Rickettsial Infection

##### 1. Studies in transmission of rickettsiae from the vector to the host

Histological changes in mouse skin parasitized by larvae (chiggers) of Leptotrombidium intermedium, L. fletcheri, L. arenicola, and L. deliense were studied to evaluate their anatomical capacity to transmit Rickettsia tsutsugamushi.

Three types of stylostome formation were recognized among the different species: the epidermal stylostome formed by the larva of L. intermedium; the mesenchymal stylostome formed by the larva of L. fletcheri; and, the mixed stylostome formed by the larva of both L. arenicola and L. deliense.

Dermal inflammations related to the three types of stylostomes were histologically defined. The possible importance of stylostome characteristics to the transmission of rickettsial organisms was studied (See Annual Report, 1976-77, Dept. of Hazardous Microorganisms, DCD&I, WRAIR).

##### 2. Studies on interactions between rickettsiae and host cells in vivo

The infection cycle of Rickettsia tsutsugamushi in mouse peritoneal mesothelial cells intimately involved the host cell plasma membrane. Organisms multiplied in the cytoplasm, moved to the cell periphery and acquired a host membrane coat as they budded from the cell surface. Rickettsiae enveloped by this membrane entered other mesothelial cells, apparently by a phagocytic mechanism. Organisms escaped from the phagocytic vacuole as the vacuole membrane and host membrane coat disintegrated. Free rickettsiae replicated by binary fission in the cell cytoplasm. Rickettsial infection of mesothelial cells induced conspicuous cellular hypertrophy with increased numbers of unaltered cytoplasmic organelles. (See Annual Report, 1976-77, Dept. of Hazardous Microorganisms, DCD&I, WRAIR).

Project 3A62760A822 MILITARY INTERNAL MEDICINE

Task 01 Military Internal Medicine

Work Unit 123 Histopathologic Manifestations of Military Disease and Injuries

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Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
5. DATE PREV SURY 76 10 01	4. KIND OF SUMMARY K. Completion	5. SUMMARY SCTY U	6. WORK SECURITY U	DA OB 6503	77 09 30	DD-DR&E(AR)636
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				10. TASK AREA NUMBER 00	11. WORK UNIT NUMBER 004	12. LEVEL OF SUM a. WORK UNIT
11. TITLE (Precede with Security Classification Code) (U) Epidemiology of Hepatitis in the Military						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS 010100 Microbiology						
13. START DATE 73 07	14. ESTIMATED COMPLETION DATE 77 09	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House			
17. CONTRACT/GRANT a. DATES/EFFECTIVE: NA	EXPIRATION:	18. RESOURCES ESTIMATE FISCAL YEAR 76 77	19. PROFESSIONAL MAN YRS CURRENT 2 2	20. FUNDS (in thousands) 11 22		
b. NUMBER: c. TYPE: d. KIND OF AWARD:	d. AMOUNT: e. CUM. AMT.					
21. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research ADDRESS: Washington, D.C. 20012	22. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Division of Preventive Medicine ADDRESS: Washington, D.C. 20012					
RESPONSIBLE INDIVIDUAL NAME: Raptund, Garrison, COL, MC TELEPHONE: 202-576-3551	PRINCIPAL INVESTIGATOR (Furnish SEAN if U.S. Academic Institution) NAME: Segal, Herbert E., LTC, MC TELEPHONE: SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]					
23. GENERAL USE Foreign intelligence not considered	ASSOCIATE INVESTIGATORS NAME: Nowosiwsky, Taras, COL, MC NAME: [REDACTED]					
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Epidemiology; (U) Hepatitis B; (U) Liver; (U) Virus Diseases						
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) To define and study the prevalence, incidence, and variables of hepatitis transmission in medical care provider and line military populations. To apply this information to the design of hepatitis prevention and control programs. 24. (U) Contemporary epidemiologic methods are employed. Multidisciplinary collaborative approaches are utilized and new methods developed as required. 25. (U) 76 10-77 09 The prevalence of Hepatitis B surface antigen and antibody and the association of seropositivity with selected variables were studied in a cohort of Army Medical Department officer personnel. Evidence of infection was found in 5 percent of personnel and was associated with several variables (WRAIR Annual Progress Report, 1974-1975, pp 960-967; Am. J. Public Health 55:667-671, 1976). Near the second anniversary of their enrollment in the preceding study, each officer was requested to complete a questionnaire and supply a blood specimen for Hepatitis B antigen and antibody testing. Serologic results and the association between seropositivity and selected variables have been reported (WRAIR Annual Progress Report, 1975-1976, pp 907-912). A group of 2,333 soldiers newly assigned to Fort Hood were studied by obtaining blood samples every four months for one year. This population was tested for Hepatitis B surface antigen and antibody, and antibody to core antigen. Data from this study have been reported (J. Inf Dis. 136:31-36, 1977) and provided valuable information regarding the use of serologic tests in epidemiologic studies of hepatitis. Two hepatitis outbreaks were studied and data obtained have been reported (WRAIR Annual Progress Report, 1974-1975, pp 970-985; Military Medicine, March 1977, pp 190-193; Military Medicine, September 1977, pp 693-695). These studies of outbreaks demonstrated the utility of contemporary epidemiologic methods coupled with available serologic tests in delineating hepatitis outbreaks and thereby providing data required to institute meaningful control programs. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76-30 Sep 77.						
Available to contractors upon contractee's approval						

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1 MAR 68

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M62770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Task 00 Military Preventive Medicine

Work Unit 004 Epidemiology of hepatitis in the military

Investigators:

Principal: LTC Herbert E. Segal, MC

Associate: COL Taras Nowosiwsky, MC; SSG Michael C. Callahan,  
L. Charlene Evans

Epidemiology of Hepatitis in the Military

Studies conducted under this work unit have been completed. Data collected have been presented in the 1974-1975 WRAIR Annual Progress Report (pp 960-967 and pp 970-985), the 1975-1976 WRAIR Annual Progress Report (pp 907-912), and four publications in the scientific literature. (This work is complementary to work described under DA OB 6513, Work Unit 135, entitled "Mechanisms of Transmission of Hepatitis Viruses.")

Project 3M62770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Task 00 Military Preventive Medicine

Work Unit 004 Epidemiology of hepatitis in the military

Literature Cited.

Publications:

1. Segal, H. E., Llewellyn, C. H., Irwin, G., Bancroft, W. H., Boe, G. D., Balaban, D. J. Hepatitis B Antigen and Antibody in the U. S. Army, Prevalence in Health Care Personnel. AJPH, 66: 667-671, July 1976.
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4. Nowosiwsky, T., Ferguson, J. A., Irwin, G. R., Randall, D. H. Hepatitis A in Military Families: Anatomy of An Epidemic. Military Medicine, 693-695, September 1977.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>*</sup>	2. DATE OF SUMMARY <sup>*</sup>	REPORT CONTROL SYMBOL	
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11. TITLE (Proceed with Security Classification Code) <sup>*</sup> <b>(U) Gastrointestinal Diseases of Military Importance</b>							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS 010100 Microbiology 008800 Life Support 002600 Biology							
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17. CONTRACT/GANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				b. PRECEDING		d. FUNDS (In thousands)	
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19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D.C. 20012				Division of Medicine			
RESPONSIBLE INDIVIDUAL NAME: RAPMUND, COL, MC, G. TELEPHONE: (202) 576-3551				ADDRESS: Washington, D.C. 20012			
PRINCIPAL INVESTIGATOR (FURNISH SSAN IF U.S. Academic Institution) NAME: BOEDEKER, MAJ, E.C. TELEPHONE: (202) 576-2582 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]							
ASSOCIATE INVESTIGATORS NAME: CHENEY, CPT, C.P. NAME: DUNN, MAJ, M.A. D.A.							
22. KEYWORDS (Proceed BACK with Security Classification Code) (U) Diarrhea (U) Absorption (U) Immunology (U) E.Coli (U) Cell Surface (U) Bacterial Adhesiveness (U) Liver Fibrosis (U) Schistosomiasis							
23. TECHNICAL OBJECTIVE, <sup>*</sup> 24. APPROACH, 25. PROGRESS (Purush individual paragraphs, identified by number. Proceed back with Security Classification Code.) 23 (U) Research efforts in the Department will continue to be directed toward Gastro Intestinal diseases of military importance. In particular, the focus is on enteropathogenic bacterial diarrheal disease including pathogenic E.Coli and Salmonellosis and Shigellosis. These have critical military relevance since they represent a major factor in troop mobility. One investigator is studying hepatic fibrosis in schistosomiasis. 24 (U) Studies of bacterial diarrhea are being conducted in three general areas 1) Pharmacologic modification of effects of infections on intestinal transport. 2) The intestinal cell surface as a determinant of bacterial colonization. 3) Cellular immune response to intestinal infection. These studies utilize several in vivo and in vitro models including in vivo intestinal perfusions of rabbits and rats, rat ileal loop models, Ussing chamber studies and in vitro agglutination of intestinal membrane fractions. Isolation and functional characterization of intestinal lymphocytes in infection is studied. 25 (U) 76-10-77 09 Transport-previous studies in this unit have shown that methylprednisolone (MP) increases mucosal water and electrolyte absorption and Na-K-ATPase activity in rat intestine in vivo without affecting adenylate cyclase. By this mechanism MP can prevent and reverse cholera toxin secretion. These results are being confirmed and extended in in vitro studies of bidirectional flux in the Ussing chamber. Cell membranes utilizing a model of adherence of pathogenic E.Coli to the rabbit intestinal surface, correlation of in vitro adherence to the brush border with colonization has been observed. The mucosal receptor for these bacteria has been solubilized and purification is proceeding. Immunology: Intestinal lymphocytes have been isolated from tissue of patients with inflammatory disease of the intestine and characterized functionally by established techniques. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 July 76 - 30 Sept 77.							
26. Authority to contract upon originator's approval 773							

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Task 00 Military Internal Medicine

Work Unit 005 Gastrointestinal Diseases of Military Importance

**Investigators**

Principal: Edgar C. Boedeker, MAJ, MC  
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Richard P. MacDermott, MAJ, MC; Edward A. Swabb, CPT, MC;  
Yuan-Heng Tai, Ph.D.; Ilja J. Weinrieb, MAJ, MC; James A.  
Wright, CPT, MC.

**Description**

The research activities in this Department have continued to focus on the toxigenic and/or invasive enteropathogenic diarrheal diseases caused by *Salmonella*, *Shigella*, *E.coli* and, to a lesser extent, *Cholera*. Three basic sets of questions are being asked about the pathogenesis of these diseases.

a. Pathophysiology of Intestinal Transport - What enzymatic and hormonal mechanisms mediate bacterial-induced secretion of water and electrolytes by the intestine? What are the normal mechanisms for salt and water absorption? How do absorptive and secretory mechanisms interact? Do they share any pathways? Can pharmacologic agents increase salt and water absorption in the face of infection or decrease secretion induced by bacterial toxins?

b. Role of Host Immune Mechanisms - How do disease causing bacteria interact with the normal cellular (lymphocyte, macrophage, polymorpho-nuclear leukocyte) and humoral (antibody) immune defense mechanisms in the intestine? What are the most effective means of inducing active cellular and humoral immunity to enteric infection (i.e., how can an effective vaccine for enteric infection be produced? What factors (antibody, complement) enable lymphocytes to kill enteric bacteria in vitro? Are these factors of equal importance in an in vivo model of enteric infection?

c. Role of Intestinal Surface Characteristics - How do disease causing bacteria interact with the surface of the gastrointestinal tract? What characteristics of this intestinal surface permit disease causing bacteria to adhere to and colonize the small intestine? What characteristics of the intestinal surface permit specific interactions with toxins produced by bacteria. Can specific interactions of bacteria or their toxins be altered by orally administered agents?

**PROGRESS AND RESULTS:**

To increase our capacity to measure Na+-K+-activated adenosine triphosphatase (Na-K-ATPase) activity in large numbers of samples, we developed

an automated ATPase assay. We utilized Technicon AA II technology and AA I components to measure inorganic phosphate colorimetrically. ATP was hydrolyzed by a membrane rich fraction of rat intestinal mucosa in duplicate manifolds in the presence and absence of potassium ions. Samples were assayed at 60/hr with a highdegree of precision and essentially no sample carryover. Absorbance was linear between 1 and 25 mM phosphate; and the amount of phosphate hydrolyzed by Na-K-ATPase and Mg-ATPase was linear with protein from 40 to 220 g/ml. Specific activities of these enzymes measured with this automated system reflected trends to those obtained with our manual ATPase assay. This automated assay has more than quadruple the output capacity of both our manual methods and other automated systems, with an equivalent degree of specificity, accuracy, and precision.(13)

We have previously shown that in vivo activation of intestinal Na-K-ATPase is associated with increased Na and water absorption. However, a role for Na-K-ATPase in intestinal Na and water absorption under basal conditions has not been demonstrated in vivo. Perfusion with 19.2mM ouabain produced net Na and water secretion and a 32% reduction in Na-K-ATPase activity ( $18.5 \pm 1.5$  vs  $12.5 \pm 1.4$ umol Pi/mg protein/h, p 0.001). Ouabain perfusion did not alter the activity of Mg-ATPase or adenylate cyclase, or affect histology. When a Ringer's-HCO<sub>3</sub> solution containing 15mM glucose was perfused, ouabain reduced glucose absorption by 50% ( $4.1 \pm 0.7$  vs  $1.9 \pm 0.3$ umol/20 min/cm, p 0.01). Residual glucose absorption induced the usual increment in Na and water absorption. As predicted from the known competition between ouabain and K for a Na-K-ATPase receptor site in vitro, ouabain-induced intestinal secretion and Na-K-ATPase inhibition could be reversed with a Ringer's-HCO<sub>3</sub> solution containing 25mM KC1. These results suggest 1) that a model for the selective in vivo inhibition of Na-K-ATPase has been developed; 2) that intestinal Na-K-ATPase plays an important role in the absorption of Na and water under basal conditions; 3) that an underlying intestinal secretory process exists as revealed by the selective in vivo inhibition of intestinal absorption; and 4) that basal Na-K-ATPase activity may be rate-limiting for intestinal glucose absorption. (1,14)

Experiments were designed to determine whether the enterotoxins of Vibrio cholerae, Escherichia coli, and Shigella dysenteriae type 1 after the movement of fluid and electrolytes in the rat cecum. Net secretion of water and sodium were observed after incubation of  $1.67 \times 10^{-5}$  g of purified cholera toxin (choleragen)/ml for 18 hr or of 50 g.ml for 3 hr. The effect of choleragen on cecal transport of water and electrolytes was related to the dose. In addition, choleragen increased cecal mucosal content of adenosine 3':5'-cyclic phosphate but did not alter the histology of the cecum. The results demonstrate that the colon responds to choleragen in a manner similar to that of other tissues. In contrast, the enterotoxins of both E.coli and S. dysenteriae type I failed to affect cecal transport of water and electrolytes. These observations may explain several phenomena associated with the diarrhea produced by bacterial enterotoxins. (15)

The effect of 3 days of methylprednisolone (MP) treatment on ion transport in rat ileum was studied in vitro. Unidirectional fluxes of Na, K, and Cl were performed on unstripped rat ileum from control and MP treated animals in the short-circuited state. In Ringer's-HCO<sub>3</sub> solution, the short-circuit current (Isc) of normal rat ileum exceeded net Na absorption; and net secretion of K, Cl and HCO<sub>3</sub> (residual ion flux) occurred. Glucose addition increased Isc and potential difference (PD) with the change in Isc accounted for by an increase in net Na absorption. MP treatment increased Isc, PD, net Na absorption and net Cl secretion and reversed the residual ion flux but did not change tissue conductance. The effect of MP treatment on Isc and net Na transport was similar to that caused by glucose and when glucose was added to MP treated ileum, the effects were additive. The major effect of MP treatment on ion transport in rat ileum studied seen in vivo. The significance of the increase in Cl secretion and reversal of HCO<sub>3</sub> transport by MP is not clear but may represent reversal of the usual CL-HCO<sub>3</sub> exchange. (2,16)

Na and Cl fluxes and short-circuit current (Isc) in rabbit ileum have been studied as a function of ionic concentrations in HCO<sub>3</sub>-free solutions. Both net Na flux ( $J_{\text{Na}}^{\text{net}}$ ) and Isc shown similar saturation functions of (Na) at fixed (Cl). They show no significantly greater than the  $J_{\text{Na}}^{\text{net}}$ . Net Cl transport, secretion, is observed only at 140mM Na and is approximately equivalent to the difference between the Isc and  $J_{\text{Na}}^{\text{net}}$ . The transcellular mucosa-to-serosa Na fluxes measured at 140 and 70mM Na do not differ significantly from the corresponding Isc. The net Cl flux varies with (Cl) at fixed (Na) while Isc is virtually not affected by (Cl). These results suggest that the absorptive Na transport process is electrogenic and responsible for the Isc and that the secretory fluxes of Na and Cl are coupled, required high (Na), vary with (Cl), and do not contribute to Isc. K-free solution abolished the Isc after a prolonged lag. Finally, the effect of a low resistance shunt pathway on active Na absorption is examined with a four-compartment model. (17)

The diarrhea observed in patients with cholera is known to be related to secretion of water and electrolytes into the intestinal lumen. However, the exact mechanisms involved in these secretory processes have remained unclear. Although it is clear that purified toxin acts on epithelial cell metabolism, its activity on Na<sup>+</sup> transport across intestinal mucosa is equivocal; reported either to prevent net Na<sup>+</sup> from serosa to mucosa. Since total transmural Na<sup>+</sup> fluxes across "leaky" epithelia involve very significant movement via a paracellular shunt pathway, we studied the effects of cholera toxin on the cellular and paracellular pathways of Na<sup>+</sup> movement. Unidirectional Na<sup>+</sup> fluxes were examined as functions of applied potential in control tissues and in tissues from the same animal treated with purified cholera toxin. Treatment of rabbit ileum in vitro with toxin stimulated the cellular component of serosa-to-mucosa Na<sup>+</sup> flux (from 2.41±0.49 equiv./h per cm<sup>2</sup> under control conditions to 4.71±0.43 equiv./h per cm<sup>2</sup> after treatment with toxin, P 0.01). The effect of cholera toxin on Na<sup>+</sup> movement through the cells from mucosa to serosa appeared to be insignifi-

cant. Finally, a marked decreased in the  $\text{Na}^+$  of the paracellular shut pathway were observed following treatment with cholera toxin. These results provide direct evidence for the hypothesis that purified cholera toxin stimulates active sodium secretion but has minimal effect on sodium absorption. (18)

The cecum of the germ free (gf) rat is filled with a large volume of liquid; cecum plus cecal contents comprise up to 25% of the rodent's body weight. In contrast, cecal contents of the conventional rat (c) are more solid and smaller in volume. To explain the presence of the liquid cecal contents in the gf rats ceceal transport of HOH and electrolytes was studied using a closed loop technique with  $^{14}\text{C}$ -polyethylene glycol as a non-absorbable marker. When  $\text{NaCl}$  ( $154\text{mEq}/11$ ) was instilled into gf rat ceca, absorption of HOH ( $9.5 \pm 3.0 \text{ ul}/\text{min/g dry weight}$ ), Na and Cl occurred; in contrast, when an equal volume of supernatant from gf cecal contents was instilled into gf rat ceca, secretion of water ( $-21.0 \pm 1.6$ ), Na and Cl occurred. Similary, when  $\text{NaCl}$  ( $154\text{mEq}/1$ ) was instilled into ceca of c rats, absorption of HOH ( $11.7 \pm 2.6$ ), Na and equal to that seen in the gf rat occurred; and when supernatant from gf cecal contents was instilled into ceca of c rats, secretion of HOH ( $-20.4 \pm 2.5$ ), Na and Cl equal to that seen in the gf rat occurred. Therefore, it appears that the composition of the gf cecal contents was responsible for the cecal secretion. Analysis of the gf cecal supernatant to determine what caused the cecal secretion revealed: very low measureable anion ( $\text{Cl}^- 2 \text{ mEq}/1$ ,  $\text{HCO}_3^- 2$ ); slight hyperosmolarity as determined by freezing point depression ( $330\text{mOsm/kg}$ ); and elevated colloid osmotic pressure. Fluid made up with  $\text{Na}_2\text{SO}_4$  to resemble the gf cecal supernatant in ionic composition and absence of exchangeable anion produced cecal secretion equal to that of gf cecal contents; fluid simulated to reproduce the effects of the colligative and colloid osmotic pressure with mannitol and 10% polyvinyl-pyrrolidone respectively had minimal effects on cecal transport. We conclude that: 1) cecal enlargement in the gf rat is associated with cecal secretion of HOH and electrolytes; 2) gf ceca absorb HOH and electrolytes in a normal fashion; 3) cecal secretion in the gf rat is due primarily to the absence of exchangeable anion in the gf cecal contents; 4) gf cecal secretion is related to an abnormality in luminal contents and is not an example of active electrolyte secretion. (3,19)

Uncertainty remains regarding the significance and mechanism of changes in intestinal HOH and electrolyte transport induced by the B-adrenergic receptor blocker propranolol (P). These studies were designed to determine the effect of P on a well-defined intestinal secretory process. HOH and electrolyte secretion and increased adenylate cyclase (AC) activity were observed in (CT) inoculated ileum and colon, but  $\text{Na-K-ATPase}$ ,  $\text{Mg-ATPase}$  and cyclic nucleotide phosphodiesterase (PDE) were unaffected. when dL-P( $4\text{mg}/\text{kg}$ )was injected daily for 3 days before loop inoculation, P did not affect HOH or electrolyte absorption or mucosal AC,  $\text{Na-K-ATPase}$  or PDE activities in saline inoculated ileal or colonic loops. However, dP-P significantly decrease CT-induced increases

in AC in control and P treated animals were similar. D-P has the non-specific membrane-blocking effects of P but is not a B-blocker. Pre-treatment with d-p (4mg/kg) for 3 days did not affect CT-induced secretion. To document that the P inhibition of secretion occurred after AC activation, a model of ileal secretion induced by dibutyryl cAMP (DB) was developed. Ileal perfusion with DB caused net HOH secretion in controls whereas in animals treated with dl-P(4mg/kg) for 3 days, DB-induced HOH secretion was significantly decreased. These results indicate that: 1) dl-P is able to inhibit CT-induced ileal and colonic HOH secretion in the rat; 2) this effect is not associated with inhibition of CT activation of AC and does not involve the non-specific membrane-blocking properties of P; 3) this inhibition apparently involves a step in intestinal secretion following AC activation. (4,20)

The hormone serotonin (5-hydroxytryptamine) has been implicated as the cause of the diarrhea seen in many patients with the carcinoid syndrome. To determine whether serotonin is an intestinal secretagogue, the effect of serotonin on intestinal water and electrolyte transport was evaluated in the rabbit. Two weeks of daily subcutaneous injection of serotonin suspended in oil resulted in a blood serotonin level elevated to twice that of controls. Intestinal transport was studied in vivo by a perfusion technique. Serotonin treatment resulted in ileal secretion and decreased mid-jejunal absorption of water and electrolytes but did not effect water absorption of D-glucose and the amino acid L-tryptophan and glucose-dependent water and electrolyte absorption were normal in serotonin-treated animals. Serotonin-induced ileal secretion was reversed by methysergide, a peripheral antagonist of serotonin action. No alterations in intestinal histology or permeability occurred in serotonin treated animals. Serotonin-induced Intestinal secretion was not associated with alterations in the activities of intestinal mucosal adenylate cyclase, cyclic nucleotide phosphodiesterase, or Na-K-ATPase. (21)

The four unidirectional fluxes of alanine across the mucosal and serosal borders of rabbit ileum were evaluated as functions of the alanine concentration on a single piece of tissue. The effects of Na<sup>+</sup> removal and of ouabain on these fluxes were investigated. Alanine was actively transported across the mucosal membrane under control conditions; Na<sup>+</sup> removal or ouabain inhibited this process as a result of a decrease in flux from the mucosal solution to the cell and an increase in the flux in the opposite direction. The results concerning mucosal efflux of alanine are apparently inconsistent with the carrier model for alanine transport at this border. Alanine transfer across the serosal membrane appeared to involve a facilitated transfer mechanism. Alanine movement at the serosal side of the cell was not influenced by Na<sup>+</sup>. (22)

The pathogenesis of Salmonella diarrhea is unclear. Bacterial invasion of the ileal and colonic mucosa resulting in an intense ileocolitis regularly occurs in concert with secretion of water and sodium in jejunum, ileum, and colon. To examine the role of alter permeability in Salmonella

diarrhea we studied intestinal histology, water and electrolyte transport, clearance of intravenously injected (<sup>14</sup>C)erythritol and (<sup>3</sup>H)mannitol, and changes in transmural electrical potential difference in normal and *Salmonella*-infected rhesus monkeys. In normal animals, absorption of water and sodium occurred in jejunum ileum, and colon and a gradient of diminishing permeability from jejunum to ileum to colon for both erythritol and mannitol was observed. Permeability as measured by determining permeability coefficients was not increased by *Salmonella* infection and in fact was significantly reduced for erythritol in the jejunum of infected animals. Perfusion with hypertonic erythritol and mannitol produced the same streaming potentials (APD) in control and infected animals, indicating no differences in transmucosal permeability. As a positive control, perfusion with 25 mM ethylenediaminetetraacetic acid in normal animals increased permeability, resulting in increased plasma-to-lumen isotope flux and no APD in response to hypertonic perfusates. These data show that despite severe alterations in intestinal histology, transmucosal permeability remains unchanged and thus is not a contributing factor in *Salmonella* diarrhea. (23)

Shigella flexneri 2a is an invasive enteric pathogen that may produce diarrhea when ingested by human beings and subhuman primates. We have previously shown that shigella diarrhea correlates with water and electrolyte transport abnormalities in the jejunum and colon. Dysentery alone is associated only with colonic transport abnormalities. To define the relationship between invasion and inflammation of the colon and the occurrence of jejunal transport abnormalities, we studied water and electrolyte transport, histology, and bacteriology in rhesus monkeys that were infected by introducing *S. flexneri* 2a directly into the cecum. In contrast to the pattern of disease seen after oral administration, cecal inoculation resulted in clinical disease in 64% of animals, of which 94% manifested dysentery alone, rarely preceded by mild diarrhea. Histologically, invasion and inflammation was limited to the colon. Secretion of water and sodium occurred in the colon of infected monkeys when compared with controls, whereas transport was normal in the jejunum and ileum. These data further demonstrate that severe dysentery can result from cecal injection of shigellae, but also suggest that the occurrence of watery diarrhea requires and may result from an undefined interaction between the jejunal mucosa and the organisms during transit through the small intestine. (24)

Atologous systems are of great interest in the study of cellular cytotoxicity. By eliminating considerations of previous allogeneic sensitization, an autologous system allows straight forward study of effector mechanisms. The induction of lymphocytotoxicity by a variety of plant lectins toward xenogeneic, allogeneic, or syngeneic target cells has been described. Although early studies of lectin-induced lymphocytotoxicity demonstrated a close correlation between the degree of mitogenic stimulation of the lymphocytes and subsequent cytotoxicity, recent work has suggested that the phenomena of mitogenesis and of cytotoxicity may be independent of one another. The ability to fractionate phytohemagglutinin into its major components, and the existence of other plant lectins that are nonmito-

genic, provided an opportunity to study human autologous cellular cytotoxicity induced by lectins, with particular attention directed to the relationship between cytotoxicity and mitogenicity. We have compared the ability of the mitogenic components of phytohemagglutinin (PHA) and the nonmitogenic lectin, wheat germ agglutinin (WGA), to cause autologous cellular cytotoxicity of human red blood cells (RBC). Both WGA and E-PHA, but not L-PHA, were found to induce lymphocytotoxicity of autologous human RBC. These findings, which show induction of cytotoxicity by WGA, a nonmitogenic lectin, but not by L-PHA, a mitogenic lectin, add to the evidence that cytotoxic capacity is independent of mitogenic capability. Furthermore, the observation that E-PHA was able to induce cytotoxicity but L-PHA was not indicates that the observed killing is not merely a nonspecific phenomenon secondary to mitogenic stimulation of the lymphocytes. (25)

We have investigated mechanisms by which plant lectins induce human peripheral blood mononuclear cells to kill red blood cells (RBC) from different species selectively. Cytotoxicity was induced by both mitogenic components of phytohemagglutinin-P (PHA), erythroagglutinating (E-PHA) and leukoagglutinating (L-PHA), and the nonmitogenic lectin wheat germ agglutinin (WGA). The target cells used in an overnight chromium release assay included human autologous RBC, human allogeneic RBC, and xenogeneic RBC from sheep or chickens. Although E-PHA induced cytotoxicity for all cell types, L-PHA caused human mononuclear cells to kill only xenogeneic RBC and, conversely, WGA induced killing of only human RBC. These differences allowed further investigation of possible control mechanisms. The target cell specificity associated with lectin-induced cellular cytotoxicity did not correlate with lectin binding to, or agglutination of, the different red blood cell types. Furthermore, preincubation of RBC with lectins followed by washing did not result in cytotoxicity. However, when the mononuclear cells were preincubated with the lectins, the same cytotoxic specificity was observed as when lectins were present during the entire assay. These experiments suggest that the target cell specificity observed with lectin-induced cellular cytotoxicity is related to a prearmed lymphocyte which seeks out and kills the appropriate target cell. (26)

Lymphocytes stimulated in vitro with plant mitogens or specific antigens release a large percentage of newly synthesized DNA into the culture media. DNA excretion cannot be accounted for solely on the basis of cell death. The possibility arises that by lymphocyte excreted DNA may transfer information and serve in a communicative role in cell-cell interactions among various lymphocyte subpopulations. We have utilized purified human lymphocyte subpopulations stimulated with phytohemagglutinin (PHA) to determine whether or not DNA excretion is a property unique to a distinct lymphocyte subgroup. Results indicate that DNA excretion is a general property of all lymphocyte subpopulations stimulated to undergo DNA synthesis by plant mitogens. (27)

Bone marrow-derived lymphocytes (B-cells) in human peripheral blood have

been shown to possess surface receptors for complement and the Fc portion of immunoglobulin molecules, membrane alloantigens and cell surface immunoglobulin. Of these cell surface markers, the presence of surface immunoglobulin is the most specific in distinguishing B cells from other leukocyte subpopulations. This led to the development of a technique of sephadex anti-Fab immunoabsorbent fractionation for the purification of peripheral blood lymphocyte subpopulations. Recently modifications to the original methodology have been described and reported. (28)

Ionophore A23187, an antibiotic isolated from a strain of streptomyces chartrensis, selectively binds divalent cations and transports  $\text{Ca}^{++}$  across biologic membranes. A23187 has been shown to induce the occurrence of a number of calcium dependent processes. The ability of ionophore A23187 to induce cell-mediated cytotoxicity of lymphocytes for autologous human redblood cells has been described and reported (29,34)

Immune associated mechanisms may play a role in perpetuating injury of hepatocytes in chronic active liver disease (CALD). Studies were performed to determine whether antibody-dependent lymphocyte cytotoxicity (ADLC) can be seen in an in vitro system using isolated rabbit hepatocytes (IRH) as target cells. Ten normal (N) individuals, 10 chronic persistent hepatitis (CPH) patients and 10 A CLD patients served as sources of sera, and normal human lymphocyte subsets (T-enriched, Null and B) as effectors separated using an immunoabsorbent column. All sera were examined for the presence of membrane-reactive immunoglobulin on IRH and human embryonal intestinal cells (HEIC) by immunofluorescence. Only CALD sera possessed membrane-reactive IgG directed against the surface of IRH. IgA and IgM were not found. No membrane fixed antibodies from any sera were found on HEIC. ADLC was assayed by a microcytotoxicity method. Unseparated lymphocytes with or without serum from normals or CPH patients induced spontaneous cell-mediated cytotoxicity (39.5-41.0%). After pretreatment with CALD sera this increased to 65.4%. In this system, T-enriched and Null cells also exhibited SCMC (38.3-45.3%), while B cells were unable to induce a cytotoxic effect. When IRH were incubated with CALD serum,  $89.5 \pm 6.22$  of the cells were injured in the presence of normal Null cells. The cytotoxic effect of T-enriched or B cells was not modified. Thus CALD serum, but not CPH serum, contains IgG antihepatocyte surface antibodies, which are capable of inducing normal null cells to kill IRH in vitro (30)

The in vitro bactericidal capability of human peripheral blood lymphocytes, granulocytes, and monocytes against Shigella flexneri 2a antibody has been investigated. We have previously shown that granulocytes were the only cell type active alone, while in the presence of antibody S. flexneri 2a were killed by lymphocytes, monocytes and granulocytes (5). We have now utilized a sequence of anti  $F(ab')_2$ -immunoabsorbent column and rosette separation techniques to isolate human peripheral blood T, B and Null lymphocytes. In the presence of antibody, unseparated lymphocytes exhibited 34% killing. Purified B Cells did not kill Shigella in

the presence of antibody. Null cell exhibited 50% killing in conjunction with antibody. Enriched T cells exhibited 48% cytotoxicity. When the enriched T cells were further purified by rosette separation with EA rosettes to remove Fc receptor bearing T cells or contaminating null cells, the resultant purified T cell population did not kill Shigella flexneri in the presence of antibody. These studies demonstrate that normal peripheral blood Null cells and perhaps Fc receptor bearing T cells are capable of effecting antibody dependent cellular cytotoxicity of Shigella flexneri 2a in vitro (6,31). Similar studies investigating lymphocyte-mediated, antibody-dependent killing of Meningococci have been performed. (7).

Current concepts of lipid-protein interaction in membranes invoke a range of associations from strongly hydrophobic (proteins anchored deep in the phospholipid (PL) matrix) to hydrophilic or ionic (superficial proteins associated with PL head groups). To define these relationships for MVM proteins, we studied the abilities of a non-ionic detergent (Triton X-100) and the 2 anionic detergents Na-Taurocholate (TC) and Na-Deoxycholate (DC) to solubilize MVM components. Aliquots of G. Pig MVMs were treated with increasing concentrations of detergents for 1 hr at RT, pH 7.4. The mixtures were centrifuged at 100,000 X G for 1 hr. The resulting supernates and pellets were examined for total protein; PL; Alkaline Phosphatase (AP), Maltase (M) and Sucrase (S) activities; and protein electrophoretic patterns in SDS acrylamide gels. Triton (0.008% to 4.0%) had the greatest differential solubilizing effect. 0.04% Triton released 47% protein, 62% M & S activities, but only 3.9% of AP and 9.7% PL. 0.1% Triton released 64% protein, 88% M & S activities, but only 45% AP and 44% PL. Increasing Triton concentration to 4% released no more than 64% protein, although 90-95% of all 3 enzyme activities and PL were released. SDS gels confirmed the resistance of several low MW proteins to Triton solubilization. TC (1 to 32mM) was the least effective solubilizer of MVMs. 1mM TC released 8% PL, 10% protein and 1% of M, S & AP. 32mM TC released 29% of PL, but no more protein or enzyme activity. DC (1 to 32mM) was an effective solubilizer, but had less differential effect than Triton. Protein and PL solubilization increased in parallel (12% at 1mM to 82% at 32mM) and ultimately 90-95% of M, S & AP were released. At 8mM DC, 70% M, 90% S, but only 16% AP were released. The selective solubilization of M & S in excess of other membrane components by Triton, and also by DC, supports the concept that disaccharidases occupy a superficial position and are held in the MVM by weaker hydrophobic interaction than AP or other membrane proteins. (10)

Colonization of the intestine by some enteropathogenic bacteria may be related to their ability to adhere to the mucosal surface. We studied the ability of a non-invasive, pilated, type O-15 E.Coli which causes diarrhea in rabbits (RDEC-obtained from Dr. J.R. Cantey) to attach to isolated brush borders (BB) and microvillus membranes (MVM). RDEC has been shown by light, immunofluorescent and electron microscopy to colonize the intestinal mucosal surface of young rabbits. To test for in vitro adherence, RDEC E.Coli were incubated with rabbit BBs ( $1-2 \times 10^7/\text{ml}$ )

and observed by phase contrast microscopy. After 30 min at RT, adherence at high bacterial concentrations ( $10^9$  bact./ml) was demonstrated by large aggregates of BB and bacteria. At  $3 \times 10^7$  bact./ml, BB aggregation was rare but adherence of 3-6 organisms/BB was seen. Incubation at 4° delayed adherence.  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ions were not required. Formalin treatment of BB prevented RDEC adherence. No adherence to rabbit BB was noted using a variety of nonpathogenic or pathogenic strains isolated from humans. Conversely, RDEC did not adhere to BB from G.Pig or human intestine. To further localize the mucosal receptor for RDEC to the intestinal surface membrane, rabbit MVM vesicles were prepared and incubated with RDEC. In a microtiter plate assay, distinctive ring patterns of agglutination of RDEC was observed using  $10^8$  bacteria and as little as 10 ug of MVM protein. No agglutination of control bacteria occurred in this assay. Neither reaction was inhibited by a variety of monosaccharides, (mannitol, D or L-fucose, galactose), sialic acid or amino sugars. In conclusion: 1) Intestinal colonization by RDEC correlates with in vitro adherence to rabbit, but not human or G.Pig, BB and MVM. 2) A sensitive microtiter assay for MVM agglutination of adherent bacteria has been developed which may prove useful in screening enteropathogens for the ability to adhere to the intestinal mucosa. (9,32)

The Sezary syndrome is a frequently lethal disease characterized by circulating malignant cells of T cell origin. The capacity of circulating malignant lymphocytes from patients with this syndrome to synthesize immunoglobulins and to function as helper or suppressor cells regulating immunoglobulin synthesis by B lymphocytes was determined. Peripheral blood lymphocytes from normal individuals had geometric mean immunoglobulin synthetic rates of 4910 ng for IgM, 1270 ng for IgA, and 1625 ng for IgG per  $2 \times 10^6$  cells in culture with pokeweed mitogen for seven days. Purified normal B cells had geometric mean synthetic rates of 198 ng for IgM, 145 ng for IgA, and 102 ng for IgG. Leukemic cells from patients with the Sezary syndrome produced essentially no immunoglobulins. Adding normal T cells to normal B cells restored their immunoglobulin producing capacity. Leukemic cells from four of five patients tested had a similar capacity to help immunoglobulin synthesis by purified normal B cells. Additionally, Sezary cells from one patient studied induced a nearly 10-fold increase in IgA synthesis by lymphocytes from a child with ataxia-telangiectasia and selective IgA deficiency. Furthermore, these Sezary cells induced more than a 500-fold increase in IgG and IgA synthesis by lymphocytes from a child with Nezelof's syndrome. When Sezary cells were added to normal unfractionated lymphocytes, they did not suppress immunoglobulin biosynthesis. In addition, unlike the situation observed when large numbers of normal T cells were added to purified B cells, there was no depression of immunoglobulin synthesis at very high malignant T cells to B cells ratios. These data support the view that Sezary T cells did not express suppressor cell activity. The results presented in this paper suggest that neoplastic lymphocytes from the majority of patients with the Sezary syndrome originate from a subset of T cells programmed exclusively for helper-like interactions with B cells in their production of immunoglobulin molecules. (33)

The role of suppressor cells in the pathogenesis of immunodeficiency was analyzed using a technique which permits study of the differentiation of B lymphocytes into immunoglobulin synthesizing plasma cells. Lymphocytes from normals synthesized 4910 ng of IgM, 1270 ng of IgA, and 1625 ng of IgG per  $2 \times 10^6$  cells when cultured for 7 days in the presence of pokeweed mitogen. In contrast the lymphocytes from patients with common variable hypogammaglobulinemia did not synthesize significant quantities of immunoglobulin. When lymphocytes from 9 of 13 patients with common variable hypogammaglobulinemia studied were co-cultured with normal lymphocytes, the synthesis of immunoglobulin by the normal lymphocytes was depressed by 75-100%. A comparable suppression of immunoglobulin synthesis by normal lymphocytes was observed when they were co-cultured with T cells from hypogammaglobulinemic patients. These studies suggest that in some patients the disease common variable hypogammaglobulinemia may not be due to an intrinsic defect of B cells alone but may be caused or perpetuated by an abnormality of regulatory T cells that act to suppress B cell maturation and antibody production. Peripheral blood lymphocytes from myeloma patients also has a drastically reduced capacity to produce polyclonal immunoglobulins. Three of 6 myeloma patients tested had circulating mononuclear cells which suppressed immunoglobulin production by co-cultured normal lymphocytes. Purified T cells from myeloma patients did not mediate this suppressor effect. These observations suggest that one mechanism for the humoral immune deficiency observed in myeloma patients is a block of polyclonal B cell maturation by suppressor cells. (35)

Deoxycholate is often absent in bile of patients with alcoholic cirrhosis. The purpose of this study was to define the mechanism for this abnormality in bile acid metabolism in alcoholic cirrhosis. Excretion and hepatic metabolism of exogenous (<sup>14</sup>C) deoxycholate were determined, quantitative and qualitative analyses of fecal bile acids were performed, and ability of fecal bacteria to metabolize cholate to deoxycholate *in vitro* was measured both in cirrhotic patients and in controls. There was no evidence for deoxycholate malabsorption or rehydroxylation. In cirrhotic patients without biliary deoxycholate, both *in vivo* levels of deoxycholate and lithocholate in feces and *in vitro* fecal bacterial conversion of cholate to deoxycholate were significantly decreased as compared to controls. The marked decrease in 7a-dehydroxylase activity of fecal bacteria of some patients with alcoholic cirrhosis results in impaired conversion of cholate to deoxycholate and explains the lack of biliary deoxycholate in these patients. (36)

Patients with cirrhosis frequently have undetectable levels of the 2 $\alpha$  bile acid DCA in bile. In separate studies (Gastroenterology, in press) we demonstrated a defect in bacterial dehydroxylation of cholic acid (CA) in stools of cirrhotics who lack DCA. To investigate a possible contribution of decreased biliary secretion of bile acids, in particular CA, to the lack of DCA in cirrhotics, we studied the hourly secretion rates of biliary phospholipids (PL), cholesterol (C), total bile acids

(TBA) and individual bile acids in 6 stable cirrhotics and 6 controls using a perfusion technique. Although PL secretion was greater in controls than cirrhotics ( $628+/-58$  vs.  $433+/-54$  Mol/hr; p 0.05), there were no differences in C (cirrh.  $55.2+/-6.2$  vs. contr.  $62+/-21$  Mol/hr) or TBA (cirrh.  $860+/-100$  vs. contr.  $811+/-151$  Mol/hr) secretion rates in the two groups. Cirrhotics lacking DCA in their fasting bile samples (N-4) had significantly lower TBA secretion ( $624+/-156$  Mol/hr) than those with DCA (N-2,  $1186+/-62$  Mol/hr; p 0.05). This decrease in TBA secretion could not be explained by decreased CA secretion. Cirrhotics lacking DCA tended to have lower CA secretion rates ( $144.6+/-58$  Mol/hr) than controls ( $227+/-47$  Mol/hr), all cirrhotics ( $165+/-47$  Mol/hr) or cirrhotics with DCA ( $208+/-69$  Mol/hr) but the differences were not significant. Decreased TBA secretion could be explained by a decreased secretion of CA and its bacterial product DCA. Secretion rates of CA+DCA in controls ( $545+/-54$  Mol/hr) were greater than those of all cirrhotics ( $201+/-51$  Mol/hr; p 0.05), cirrhotics with DCA ( $315+/-19$  Mol/hr; p 0.01) and cirrhotics lacking DCA ( $144.6+/-5.8$  Mol/hr; p 0.01). In conclusion 1) stable cirrhotics secrete less CA+DCA than normal subjects, although TBA secretion may be normal 2) cirrhotics lacking DCA have the lowest CA+DCA secretion rates and lower TBA secretion rates than those with DCA 3) in the absence of DCA malabsorption and in the presence of defective bacterial conversion of CA to DCA these results indicate a defect in hepatic synthesis of CA in cirrhotics lacking DCA. (10,37,38)

Previous studies in rats treated with triiodothyronine showed increased percentages of CDC in bile. We examined 18 patients with HT to determine if similar abnormalities occurred in man. GLC of extracted bile provided a screening method for determination of individual bile acids (BA), Gallbladder bile, obtained by duodenal aspiration after stimulation of contraction by amino acids, was enzymatically hydrolyzed, and the free BA were analyzed as their trimethylsilyl ethers on 1% QF-1. Patients with HT exhibited increased percentages of CDC in bile (Cholic (C)  $14.8+5.2\%$ , CDC  $71.4+4.5\%$ , and Deoxycholic (D)  $10.8+4.6\%$ ) compared with controls (C  $30.8+6.0\%$ , CDC  $42.7+4.8\%$ , and D  $17.5+5.0\%$ ). Because of the predominance of CDC in bile and the fact that 8 of the 18 patients complained of pruritus, including one patient with pruritus as the chief complaint, we measured CDC levels in serum. To accurately measure low serum levels, a RIA for conjugates of CDC was developed. Glycocheno-deoxycholic acid was bound to albumin by the carbodiimide reaction and injected into rabbits at 2 week intervals for 4 months. Antisera was highly specific for CDC and the standard curve was linear from 0.1 to 2.0 mol/l. Compared to controls, patients with HT and pruritus had significant elevation of serum CDC ( $3.63+1.59$  mol/l vs  $0.33+0.04$  p 0.05). Serum CDC was highest in the patient with pruritus as the chief complaint (14.0 mol/l) and dropped to 1.0 mol/l with cessation of pruritus, after treatment for HT. These data show the complimentary use of GLC and RIA for determination of biliary and serum BA, and suggest that 1) pruritus occurs more frequently in HT than previously recognized; 2) patients with HT have

increased biliary CDC: 3) serum CDC levels are increased in patients with HT and pururitis; 4) therapy of HT is associated with resolution of pruritus and return of serum CDC levels towards normal. (11, 12, 39)

CONCLUSIONS AND RECOMMENDATIONS:

Work in this Department has shown that Na-K-ATPase is closely correlated with water and electrolyte transport in the intestine. It has been shown that pharmacological activation of Na-K-ATPase can prevent or reverse fluid and electrolyte secretion caused by cholera toxin and the adenylate cyclase/cyclic amp system. Recent work has shown that Na-K-ATPase activity of the intestine can be successfully blocked by ouabain, revealing an underlying secretory process. Similar observations have been made in an in vitro system. It appears that two separate and oppositely directed transport processes in the intestine can be modulated independently.

Other mechanisms for intestinal secretion have been investigated. It has been shown that the massive cecal secretion in the germ free rat is due primarily to lack of exchangeable anion in the cecal contents. In addition, propranolol was shown to inhibit cholera toxin induced ileal secretion, without influencing either Na-K-ATPase or cholera toxin activation of adenylate cyclase. Serotonin also influenced intestinal secretion by mechanisms independent of Na-K-ATPase or adenylate cyclase.

The mechanisms whereby human lymphocytes can be induced to be cytotoxic toward a defined cellular population (including bacterial populations) have been investigated extensively. The induction of lymphocyte toxicity by plant lectins and its relation to mitogenicity has been defined and target cell specificity ahs been demonstrated. Lectins seem to prearm lymphocytes to kill specific target cells independent of previous sensitization. In addition, the induction of cytotoxicity by modification of transmembrane Ca++ fluxes has been studied. The column technique for isolating human lymphocytes has been set up and modified. Populations of peripheral lymphocytes obtained from these columns have been used to determine their cytotoxicity for enteric pathogens (shigella) as well as host cells (hepatocytes). Collaborative studies on cytotoxicity for meningococci have been performed.

In vitro adherence of pathogenic E.Coli to rabbit intestinal cell membranes has been demonstrated. Membrane receptors for these pathogenic E.Coli have been solubilized from brush border membranes of rabbit epithelial cells using detergents. In addition, a microtiter assay for quantitating in vitro adherence of bacteria to cell membranes has been developed.

The major aim of the Department continues to be the elucidation of the fundamental mechanisms and limiting steps in normal and pathologic intestinal secretion and adsorption. It is becoming increasingly clear that infectious diarrhea is related to specific functional alterations

in intestinal cells and not to general cell destruction. These alterations usually follow the successful colonization of the host by the infecting organism. In general, bacterial adherence leads to intestinal colonization and this is followed by toxin-mediated effects on the host epithelial cells. If the cell receptors, specific toxins or host intestinal epithelial enzyme activities can be defined, specific pharmacologic reversal or interference with these mechanisms may be possible. In addition, the factors involved in host immunologic resistance to enteric bacteria is being pursued with emphasis on the mechanisms of cell killing and the subpopulations of lymphocytes involved. Current work is being directed toward: 1) obtaining intestinal lymphocytes and their subpopulations from humans and animals, 2) establishing a well-characterized model for enteric disease (using the rabbit) in which membrane receptors are known and lymphocyte populations can be defined, 3) extending the studies of bacterial adherence to human pathogens using human tissue in the assay system.

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Task 00 Military Internal Medicine

Work Unit 005 Gastrointestinal Diseases of Military Importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>b</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL		
				DA OA 6442	77 10 01	DD-DR&E(AR)636		
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10. NO./CODES: <sup>b</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER		
a. PRIMARY	62770A	3M762770A802		00		006		
b. CONTRIBUTING								
c. CONTRIBUTING	CARDS 114F							
11. TITLE (Pecede with Security Classification Code) <sup>b</sup>								
(U) Rickettsial Diseases of Military Personnel								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>b</sup>								
010100 Microbiology								
13. START DATE	14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY	16. PERFORMANCE METHOD				
55 08	CONT		DA	C. In-House				
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE				
a. DATES/EFFECTIVE:	NA	EXPIRATION:		b. PROFESSIONAL MAN YRS	d. FUNDS (In Thousands)			
d. NUMBER:				PRECEDING	4	178		
c. TYPE:		d. AMOUNT:		FISCAL YEAR	77			
e. KIND OF AWARD:		f. CUM. AMT.:		CURRENT	78	216		
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION				
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER [REDACTED]				
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS				
				NAME: Eisenberg, George H.G., Jr., MAJ, MSC				
				NAME: Nacy-Mahady, Carol, Dr.				
22. KEYWORDS (Pecede EACH with Security Classification Code) (U) Rickettsial infections; (U) Laboratory diagnosis; (U) Vaccines; (U) Epidemiology								
23. TECHNICAL OBJECTIVE. <sup>b</sup> 24. APPROACH. 25. PROGRESS (Pecede individual paragraphs identified by number. Pecede text of each with Security Classification Code.)								
23. (U) 1. Development of experimental rickettsial immunogens; 2. Pathology of rickettsial infection in laboratory animals; 3. Cellular immune response of the host to infection with rickettsiae. These studies are directly aimed at development of safe inactivated vaccines for protecting troops in the field and for development of accurate and sensitive tests to assay the extent of immunity induced by vaccination.								
24. (U) 1. Gamma irradiation of rickettsiae to produce attenuated, non-replicating organisms; 2. Evaluation of rickettsiae-mesothelial cell interaction by electron microscopy; 3. Transfer of spleen and peritoneal exudate lymphocytes from immune to recipient animals with subsequent challenge by virulent rickettsiae.								
25. (U) 76 10-77 09 1. The feasibility of gamma irradiated rickettsiae as an immuno-gen has been established. Studies are in progress to determine the duration of immunity elicited in laboratory animal models. 2. The in vivo infection of mouse mesothelial cells has been studied by electron microscopy. The complete infection cycle in these cells has been described; and the intimate association with host cell plasma membrane helps explain the difficulties experienced in purification of these organisms. 3. Spleen and peritoneal exudate lymphocytes were shown to have different protective capacities, radiosensitivity and trafficking patterns in recipient animals. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Jul 76 - 30 Sep 77.								

<sup>a</sup>Available to contractors upon originator's approval

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Project 3M762770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Work Unit 006 Rickettsial Diseases of Military Personnel

Investigators.

Principal: Joseph V. Osterman, Ph.D.; MAJ George H.G. Eisenberg, Jr., MSC; Carol Nacy-Mahady, Ph.D.

Associate: SP5 John A. Hallam; SP4 Denise G. Caron

Description.

To determine the mechanisms of rickettsial pathogenicity and to characterize both the humoral and cell-mediated immunological responses to rickettsial infection.

Progress.

I. Mechanisms of rickettsial pathogenicity.

A. Experimental infection of mouse peritoneal mesothelium with scrub typhus rickettsia: An ultrastructural study.

The intraperitoneal inoculation of susceptible mice with Rickettsia tsutsugamushi leads to a fatal infection, with pathology essentially restricted to the peritoneal cavity. The immune response is incapable of controlling the proliferation of rickettsiae and involvement of peritoneal mesothelial cells is apparent early in the course of infection. These cells exhibit swelling, rounding and vacuolation, followed by desquamation in the later stages of disease.

In vitro morphological studies on the interaction of scrub typhus rickettsiae with cultured rat fibroblasts have described a lack of degenerative changes in heavily infected cells, and the extrusion of rickettsiae by way of filamentous microfibrillar structures protruding from the surface of the cell. Subsequent studies in mouse L cells supported the concept that scrub typhus rickettsiae escaped from cells by extrusion, rather than cell rupture, and Higashi (11) suggested that as the rickettsiae left the cytoplasm and passed through the cell membrane a portion of the cytoplasmic substance remained closely adherent to the rickettsiae. More recently, scanning and transmission electron microscopy of Vero cells infected with R. tsutsugamushi reiterated the lack of cell damage and described numerous protrusions of host cell membrane which contained rickettsial organisms.

Mesothelial cells line the peritoneal cavity, are easily distinguished during microscopic observation, and are prominent in the pathology of lethal experimental infection of mice with R. tsutsugamushi. We felt they constituted an appropriate in vivo model to study the cellular pathology of rickettsial infection and to investigate the role of host cell membrane in the infection cycle of scrub typhus rickettsiae.

BALB/c mice were divided into experimental and control groups. Animals used for experimentation were infected by intraperitoneal (i.p.) injection of 1,000 MLD<sub>50</sub> of Karp in a volume of 0.2 ml. Control mice were sham-infected with an i.p. injection of a similar volume of 20% normal yolk sac.

Clinical and gross observation: Pairs of infected and control mice were sacrificed on days 1, 3, 6, 9 and 10. Infected mice developed ruffled coats by 9 days after inoculation of rickettsiae and were moribund on day 10. A moderate peritoneal exudate was present in infected animals sacrificed on day 9, and a copious, fibrinous exudate was observed in animals killed on day 10. Control mice remained healthy until sacrificed and no peritoneal exudate was observed.

Light microscopy: Mesothelial cells from infected mice showed increased cytoplasmic basophilia and enlargement which corresponded to the appearance of increased numbers of intracellular rickettsiae. Nuclei were usually enlarged and showed an open chromatin pattern (Fig. 1). In contrast, mesothelial cells from control animals maintained normal morphology through day 10 (Fig. 2).



Figure 1. Two enlarged mesothelial cells exhibit basophilic cytoplasm containing many rickettsiae 10 days after inoculation. Nuclei show an open chromatin pattern, and the one on the right is enlarged. Wright's stain; X900.

Figure 2. Mesothelial cells from sham-infected control animals 10 days after inoculation show normal morphology, with pale-staining cytoplasm and compact nuclei. Wright's stain; X900.

Electron microscopy: Mesothelial cells from infected mice killed on days 9 and 10 contained many intracellular rickettsiae. Infected cells were increased in height and showed greatly increased numbers of mitochondria, stacks of Golgi lamellae, segments of granular endoplasmic reticulum and free polyribosomes. The structure

of these cytoplasmic organelles remained unaltered. The enlarged nuclei contained one to several nucleoli, and showed a high ratio of euchromatin to heterochromatin (Fig. 3). A few heavily infected mesothelial cells detached from the basal lamina and exhibited various degenerative changes including irregular dilation of the perinuclear space, disorganization of chromatin and small breaks in the plasma membrane.

The mesothelium of control mice remained intact throughout the 10 day observation period, appearing in cross-section (Fig. 4) as a single layer of thin, flat cells with small numbers of mitochondria, an occasional Golgi apparatus, abbreviated segments of granular endoplasmic reticulum and scattered, free, single ribosomes.

Rickettsiae appeared as pleomorphic coccobacilli with mottled, granular cytoplasm containing a loose network of fine fibrils (Fig. 3). Organisms were sometimes partially separated from the cytoplasmic matrix by an irregular, narrow, electron-lucent zone. No intranuclear rickettsiae were seen.

The egress of rickettsiae from intact, infected cells is shown in Figs. 5-7. Individual organisms were located within plasma membrane evaginations at the free surfaces of mesothelial cells. The host cell plasma membrane, connected to the cell by a short stalk of cytoplasm, formed an additional coat around organisms. Rickettsiae surrounded by this additional membrane layer were also observed completely detached from host cells. In moribund mice, detached, degenerated mesothelial cells were occasionally observed in the process of disintegration and rickettsiae escaping from these cells appeared devoid of host membrane.

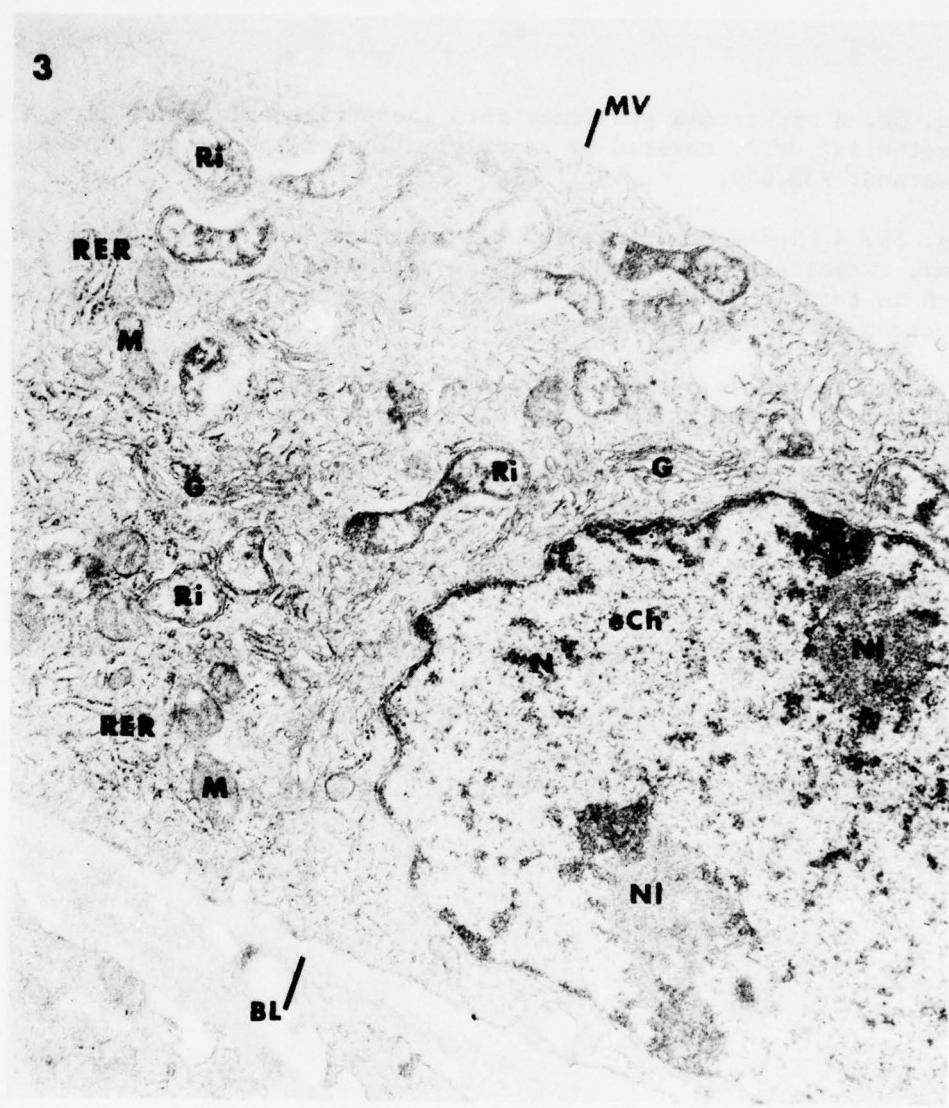
Scrub typhus organisms bearing the host-derived coat were capable of entering other mesothelial cells. Figs. 8-10 show coated rickettsiae within plasma membrane invaginations, and within membrane lined vacuoles near the free cell surface. In further observations (Figs. 11-12) the host membrane coat surrounding the rickettsiae and the vacuole membrane appeared as discontinuous, electron-dense membrane fragments encircling an intact organism. Rickettsiae showing various degrees of central annular constriction characteristic of binary fission were always free within the cytoplasm (Fig. 13). Neither direct penetration of the cell membrane by rickettsiae, nor phagocytosis of uncoated rickettsiae was observed.

The conspicuous hypertrophy of mesothelial cells infected with R. tsutsugamushi probably represented a nonspecific reaction to cellular stress, since a similar response has been described for mesothelial cells subjected to unrelated conditions such as experimental asbestosis (Suzuki). While increased in number, cytoplasmic organelles concerned with energy production or protein synthesis usually retained their normal structure, suggesting the absence of cell damage. In vitro studies of mouse lymphoblasts infected with R. tsutsugamushi have also

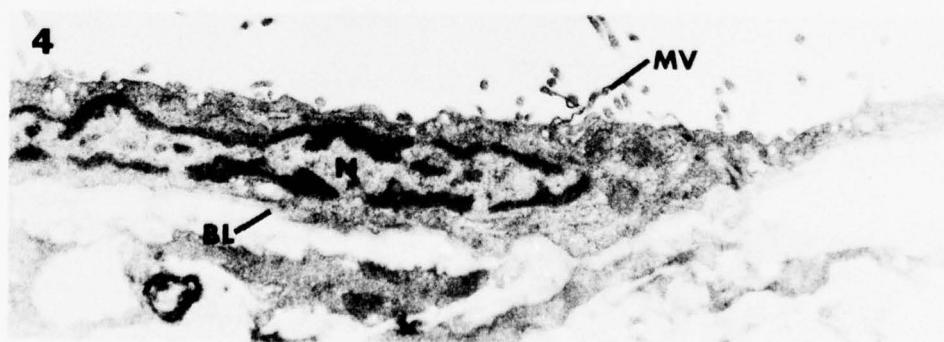
**Fig. 3.** A vertical section of an infected mesothelial cell on day 10 following inoculation shows increased height of both cytoplasm and nucleus. A number of rickettsiae (R) appear free in the cytoplasm, and some are partially bordered by a clear zone. The cytoplasm exhibits unusually large numbers of mitochondria (M) stacks of Golgi lamellae (G), segments of granular endoplasmic reticulum (RER) and free polyribosomes. The nucleus (N) shows a high ratio of euchromatin (eCh) to heterochromatin (hCh), and two nucleoli (N1) are visible. The basal lamina (BL) is intact, and a few microvilli (MV) project from the free cell surface; X17,000.

**Fig. 4.** A low, flat profile and sparse cytoplasmic organelles characterize this vertical section of a mesothelial cell from a sham-infected mouse sacrificed on day 10 postinoculation. The thin nucleus (N) exhibits heavy blocks of heterochromatin (hCh); X17,000.

3



4



**Fig. 5a.** A rickettsia protrudes into the peritoneal cavity from a mesothelial cell, covered by an evagination of the cell's plasma membrane; X33,000.

**Fig. 5b.** A higher magnification demonstrates the host membrane coat (hm), rickettsial cell wall (cw) and rickettsial cell membrane (cm), each in turn composed of two electron-dense layers separated by an electron-lucent layer; X190,000.

**Fig. 6a.** Host cell plasma membrane coats a rickettsia which is attached to a cell by a short stalk of cytoplasm; X33,000.

**Fig. 6b.** Host membrane (hm), rickettsial cell wall (cw) and rickettsial cell membrane (cm); X190,000.

**Fig. 7a.** Membrane coat surrounds an organism detached from its host cell; X33,000.

**Fig. 7b.** No fine structural changes in either the host membrane coat (hm), rickettsial cell wall (cw) or rickettsial cell membrane (cm) are apparent after separation from the host cell; X190,000.

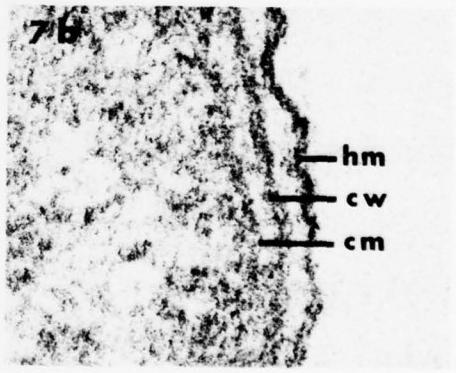
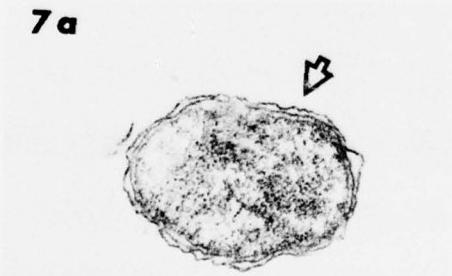
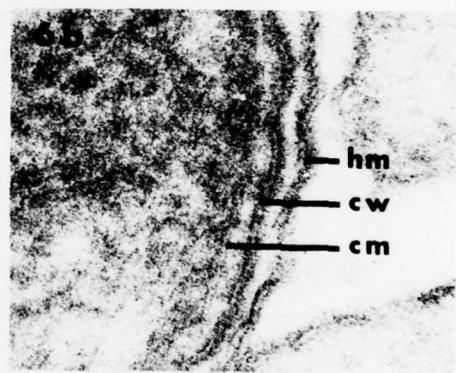
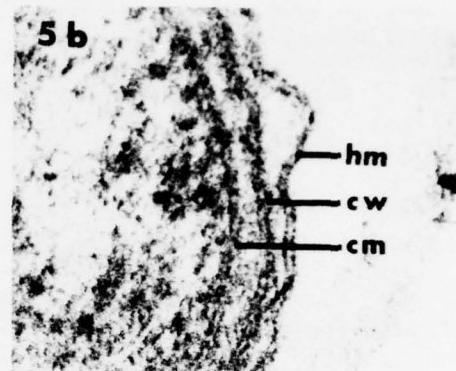
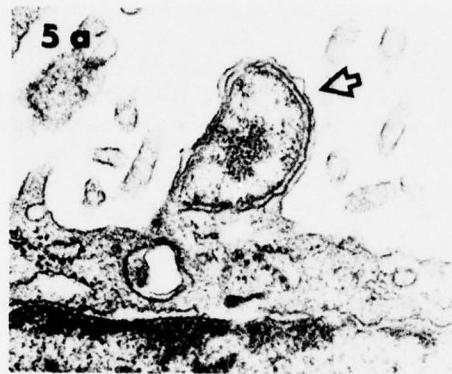


Fig. 8a. A membrane-coated rickettsia is beginning to enter a mesothelial cell; X33,000.

Fig. 8b. Detail of developing vacuole membrane (vm), host membrane coat (hm), rickettsial cell wall (cw) and rickettsial cell membrane (cm); X190,000.

Fig. 9a. A membrane-coated rickettsia lies within a membrane-lined vacuole just beneath the cell surface; X33,000.

Fig. 9b. Vacuole membrane (vm), host membrane coat (hm), rickettsial cell wall (cw) and rickettsial cell membrane (cm); X190,000.

Fig. 10a. A rickettsia appears slightly deeper in the cytoplasm, but remains within a membrane-lined vacuole; X33,000.

Fig. 10b. Vacuole membrane (vm), host membrane coat (hm), rickettsial cell wall (cw) and rickettsial cell membrane (cm); X190,000.

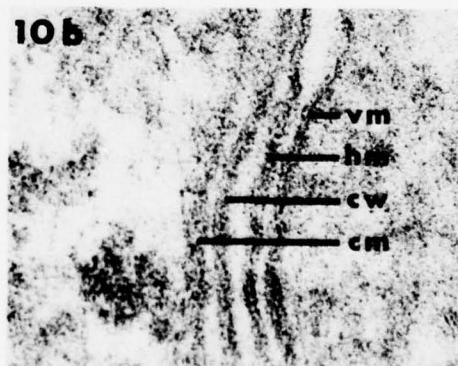
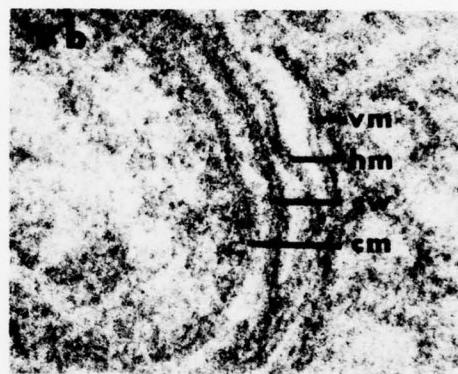
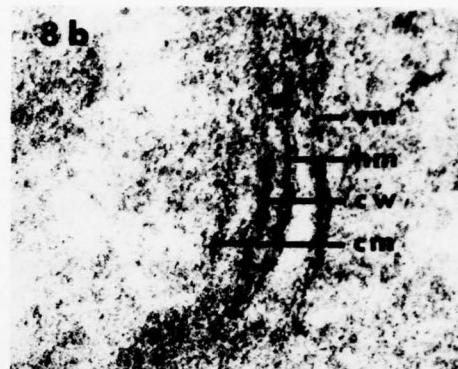


Fig. 11a. The vacuole membrane shows focal thickening and an uneven increase in electron density, while part of the host membrane coat appears tightly bound to the rickettsial cell wall; X33,000.

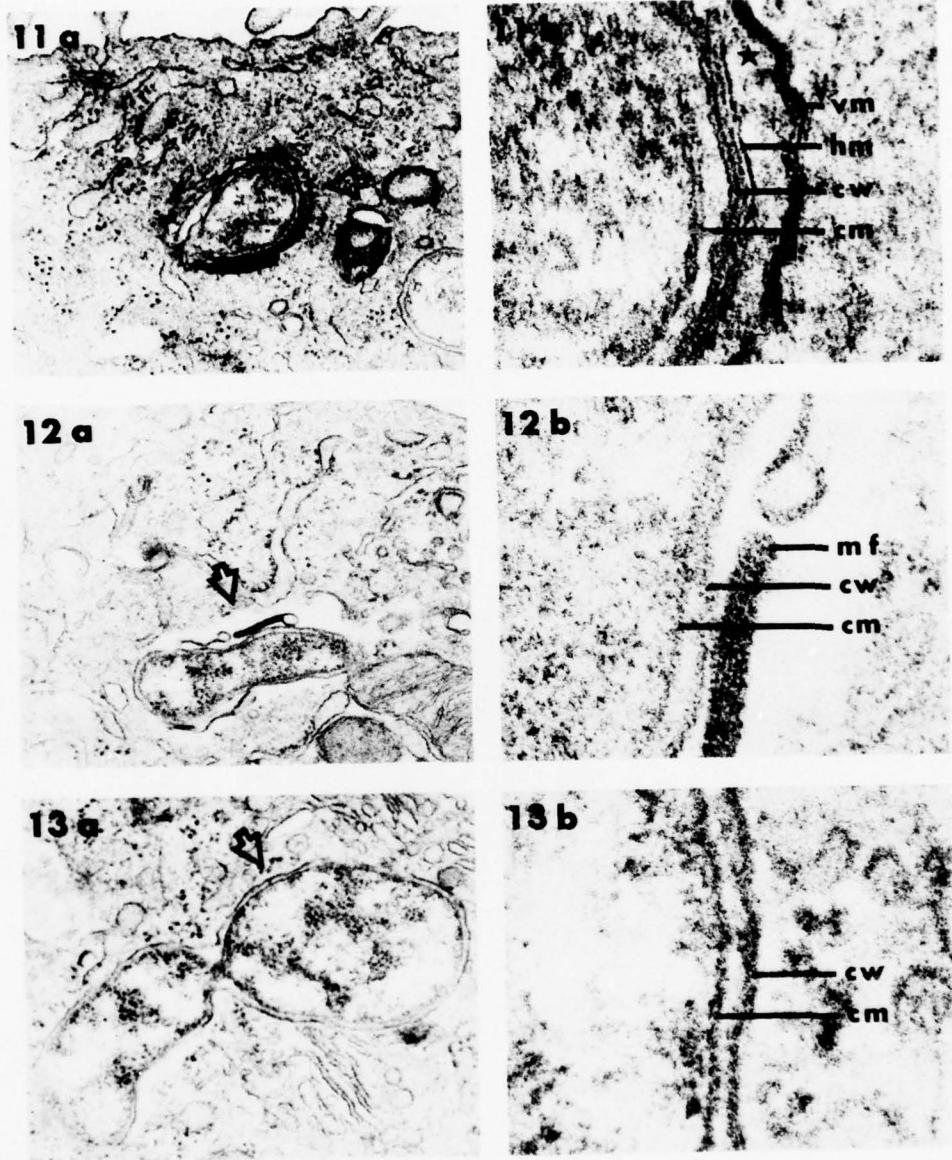
Fig. 11b. Some host cell cytoplasm (cyt) appears within the vacuole. Vacuole membrane (vm), host membrane coat (hm), rickettsial cell wall (cw) and rickettsial cell membrane (cm); X190,000.

Fig. 12a. Incompletely surrounded by a few membrane fragments and a clear zone, a rickettsia appears largely free in the mesothelial cell cytoplasm; X33,000.

Fig. 12b. Electron-dense membrane fragment (mf) lies within a clear zone, while the rickettsial cell wall (cw) and cell membrane (cm) remain intact; X190,000.

Fig. 13a. A rickettsia in the process of binary fission appears deep within the host cell cytoplasm, unencumbered by host cell membranes; X33,000.

Fig. 13b. Rickettsial cell wall (cw) and cell membrane (cm); X190,000.



suggested a lack of severe cytopathology, with no marked difference in oxygen uptake or anaerobic glycolysis between infected and control cells. Similarly, incorporation of radioactive amino acids and nucleotides by irradiated L-cells was little affected during the first few days of in vitro infection with scrub typhus organisms. However, prolonged cultivation of infected BSC-1 cells led to severe and characteristic cytopathology that was analogous to the degenerative changes we occasionally observed in mesothelial cells of moribund mice.

The host cell plasma membrane was intimately involved in the infection cycle of scrub typhus rickettsiae in mouse mesothelial cells, and our observations were compatible with the hypothetical scheme shown in Fig. 14. As rickettsiae multiplied in the cytoplasm

#### 14

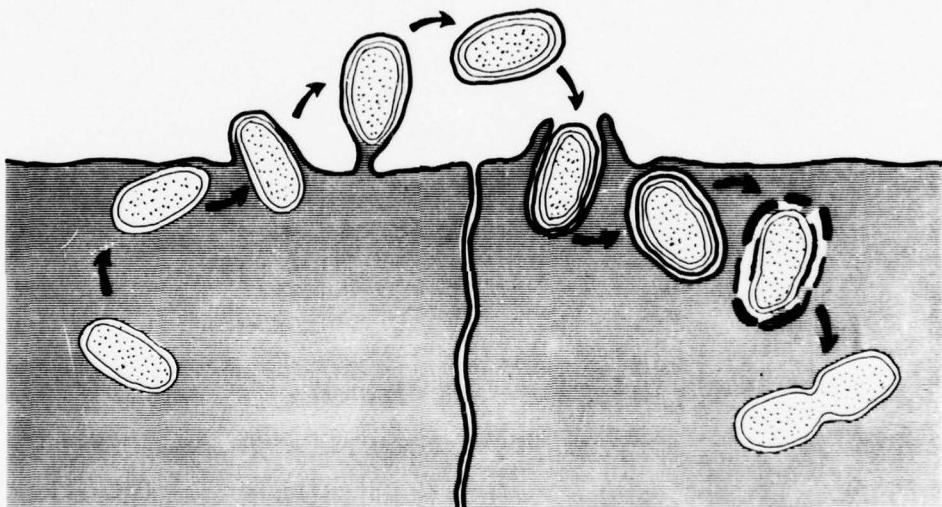


Figure 14. Hypothetical scheme for infection cycle of *R. tsutsugamushi* in mouse mesothelial cells.

of intact peritoneal mesothelial cells, some organisms moved to the cell surface, acquired a host membrane coat derived from the host cell plasma membrane and budded from the cell surface. Only in rare instances did rickettsiae appear to be released from detached, degenerated and disintegrating cells of moribund mice without acquiring a host membrane coat.

Rickettsiae encased by host-derived membrane entered other mesothelial cells, apparently by phagocytosis. Mesothelial and epithelial cells are known as "facultative phagocytes"; these cells are not ordinarily phagocytic, but are capable of initiating phagocytosis through poorly defined, nonimmunological interactions.

The presence of the host membrane coat may have been an important factor favoring phagocytosis of rickettsiae by mesothelial cells, since we did not observe phagocytosis of uncoated organisms by these cells. Also consistent with this view was the finding of Raftery (1) that mesothelial cells were apparently unable to phagocytize polystyrene spheres which were similar in size to rickettsiae.

It has been suggested that other obligate intracellular parasites such as Toxoplasma gondii and Chlamydia psittaci enter facultative phagocytes by active induction of phagocytosis. The entry of R. tsutsugamushi into guinea pig epithelial cells occurred by phagocytosis, and penetration of mouse lymphoblasts and fibroblasts was largely dependent on the viability of the rickettsiae. It is possible that induced phagocytosis, facilitated by the host membrane coat of the organisms, mediated the entry of scrub typhus rickettsiae into mouse mesothelial cells.

Following phagocytosis, the final event in the entry of R. tsutsugamushi into mesothelial cells was escape from the phagosome into the cytoplasm. This probably occurred shortly after phagocytosis, since phagosomes containing rickettsiae were never seen far from the cell surface. The host membrane coat and the phagosome membrane seemed to disintegrate and disappear at about the same time, and the organism emerged into the cytoplasm with its cell wall and cell membrane intact. Once free in the cytoplasm, organisms moved deeper into the cell and began to multiply by binary fission to repeat the infection cycle.

## II. Characterization of humoral and cell-mediated immunity to rickettsial infection.

### A. Host defenses in experimental scrub typhus: Effect of chloramphenicol.

The efficacy of chloramphenicol as a chemoprophylactic and chemotherapeutic agent in the treatment of natural scrub typhus infections in man and experimental infections of rodents has been well established. In the experimental situation, appropriate treatment of infected mice not only resulted in their survival but also rendered such animals immune to subsequent lethal challenge by homologous or heterologous strains of Rickettsia tsutsugamushi. Experimental evidence from laboratory infections of mice and circumstantial evidence from natural infections of man have suggested that reduction of the relapse rate following withdrawal of chloramphenicol therapy was related to the development of a protective immune response.

This laboratory has recently described the development of protective heterologous immunity in mice to infection with R. tsutsugamushi. We have shown that resistance in the early stages of primary scrub typhus infection was due principally to the development of cellular immunity with the role of circulating antibody remaining undefined. Considering the effectiveness of chloramphenicol in treatment of scrub typhus infections, it was of interest to study the effects of this drug on the development of immunity in primary infections. In this report, we used BALB/c mice infected with the Karp strain of R. tsutsugamushi and have correlated the length of chloramphenicol treatment and the time of initiation of treatment following infection with the subsequent development of cellular and humoral immune responses.

Chloramphenicol solution was prepared in distilled water to a final concentration of 2.5 mg/ml and given to mice as their drinking water. Two basic regimens of chloramphenicol treatment were employed: Group I: Drug was given to mice 2 days prior to Karp inoculation and maintained through the 14th day post inoculation; Group II: Drug was given 7 through 21 days post Karp inoculation. When mice were maintained on antibiotic for the full duration of each regimen complete survival was observed. Infected, untreated animals all died, usually within 10-13 days after challenge.

The duration of antibiotic treatment necessary for protection in each regimen was investigated by withdrawing the drug on successive days after initiation of treatment. Chloramphenicol was first withdrawn on the third day of treatment in Group I and after the first day of treatment in Group II.

Group I mice failed to survive if the drug was withdrawn prior to 11 days of post-infection therapy (Fig. 15). If the antibiotic was removed 11-13 days after infection, some mice did survive, and by day 14 no mortality was observed. Although ultimate death of animals was not affected by the number of days of antibiotic treatment during the initial 10 days after infection, some effect of the drug was noted, as the length of survival before death increased proportionately with the duration of drug treatment during this period (Table 1). However, subtraction of the number of days of treatment from the total days of survival reveals that the mice invariably succumbed after approximately 13 days of uninhibited rickettsial proliferation. Animals surviving antibiotic withdrawal were challenged with 1,000 MLD<sub>50</sub> of Karp 35 days after termination of treatment and found to be immune.

Some animals in Group II survived when antibiotic was withdrawn after only 2 days of treatment and complete protection was first observed 12 days after infection, or 5 days after initiation of treatment. Despite the early appearance of survivors, occasional deaths were noted in this group when the drug was withdrawn at times subsequent to initial observation of complete protection (days 17 and

19 post-infection, Figure 15). Survival time of dying mice was prolonged and survivors were resistant to subsequent challenge as observed in Group I (Table 1).

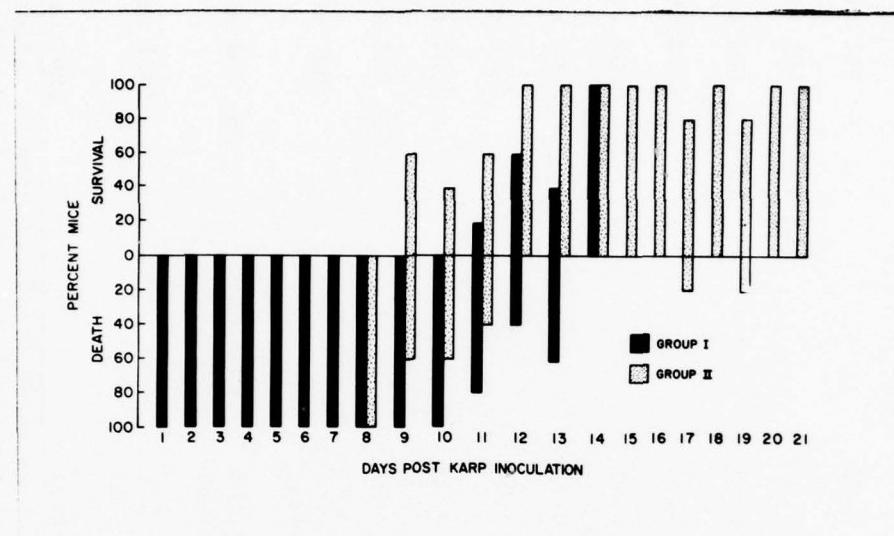


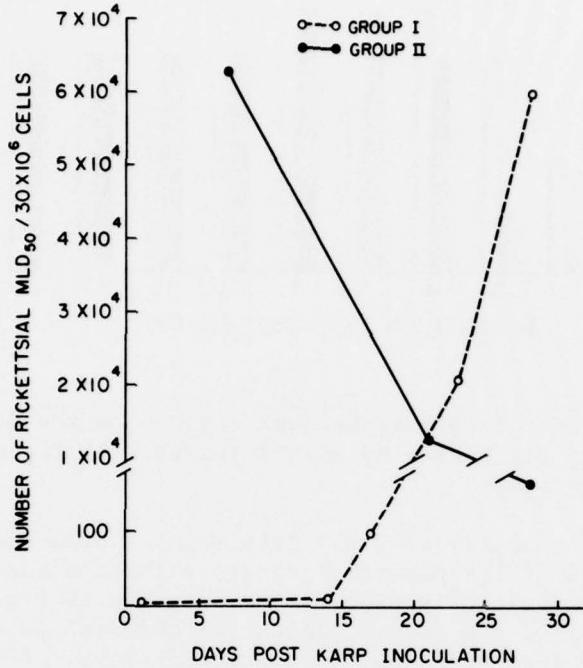
Figure 15. The effect of time of initiation of chloramphenicol treatment and duration of therapy on the ability of mice to survive infection with the Karp strain of *R. tsutsugamushi*. Chloramphenicol therapy was initiated two days prior to infection in Group I and seven days after infection in Group II. Each bar indicates the fate of a separate group of five mice that were withdrawn from drug therapy on the day indicated.

Another means of assessing the efficacy of a particular antibiotic regimen was by assay of the infectious rickettsial burden in the spleen. To investigate this effect,  $30 \times 10^6$  non-adherent spleen cells were obtained from each group of mice on selected days and briefly homogenized. The disrupted cells were transferred to recipient mice either undiluted ( $30 \times 10^6/0.2$  ml) or after serial dilution, which subsequently allowed calculation of the rickettsial  $MLD_{50}$  of the transferate. Inspection of Fig. 16 indicated a constantly increasing infectious burden in the spleens of Group I mice which reached a level of  $6.0 \times 10^4$   $MLD_{50}$  Karp/ $30 \times 10^6$  cells at 28 days post infection. Quite the opposite effect was seen in Group II mice, in which the initial antibiotic-free period of rickettsial growth was reflected in  $6.3 \times 10^4$   $MLD_{50}$  of rickettsiae recoverable at an early time but very few detectable at 28 days. It is clear that Group II, which allows an early replication period for the rickettsiae, was more effective in producing survival in the shortest period of time (see above), and also in reducing the rickettsial burden of the spleen.

Table 1. Length of survival following withdrawal of chloramphenicol<sup>a</sup>

Days of drug treatment	Group I	Group II
1	14 ( $\pm$ 0.6)	13 ( $\pm$ 0.4)
2	15 ( $\pm$ 0.0)	14, 14; 60% survival
3	16 ( $\pm$ 0.4)	12, 12, 21; 40% survival
4	18 ( $\pm$ 0.5)	13, 15; 60% survival
5	17 ( $\pm$ 1.3)	100% survival
6	20 ( $\pm$ 0.4)	100% survival
7	21 ( $\pm$ 0.2)	100% survival
8	22 ( $\pm$ 1.0)	100% survival
9	23 ( $\pm$ 0.3)	100% survival
10	23 ( $\pm$ 0.6)	26; 80% survival
11	24, 24, 24, 26; 20% survival	100% survival
12	25, 29; 60% survival	26; 80% survival
13	26, 27, 27; 40% survival	100% survival
14	100% survival	100% survival

<sup>a</sup> When all animals in each group of 5 died, the mean day of death  $\pm$  S.E. of mean is indicated.  
When some animals survive, the day of death of each of the remaining animals is listed.



**Figure 16.** The effect of chloramphenicol regimen on the number of MLD<sub>50</sub> of rickettsiae present in non-adherent spleen cells from mice infected with the Karp strain of R. tsutsugamushi. Mice in both groups were maintained on antibiotic for the full duration of each regimen and all animals survived infection.

The production of complement-fixing antibody by mice included in the 2 groups was different with respect to time of appearance in the serum and maximum titer achieved (Fig. 17). The mice in Group I required 3 weeks to develop a detectable response, but evidenced a relatively high titer (1:160) by 5 weeks and maintained antibody levels in the range of 1:80 to 1:160 through 15 weeks. Mice in Group II had a 1:20 titer 14 days after inoculation of rickettsiae and a constant increase in CF antibody level until a titer of 1:160 was achieved in the fourth week. Serum titer was reduced to 1:40 by the fifth week and remained in the range of 1:40 to 1:80 for the remainder of the 15 weeks.

The capacity of surviving mice in each treatment group to withstand rechallenge with 1,000 MLD<sub>50</sub> of Karp at time of drug withdrawal is shown in Table 2. In addition, the ability of such survivors to passively confer protection by transfer of 30 X 10<sup>6</sup> splenic lymphocytes to recipient mice, is also indicated. The Group I mice were assayed 17 days after Karp infection (3 days after completion of antibiotic

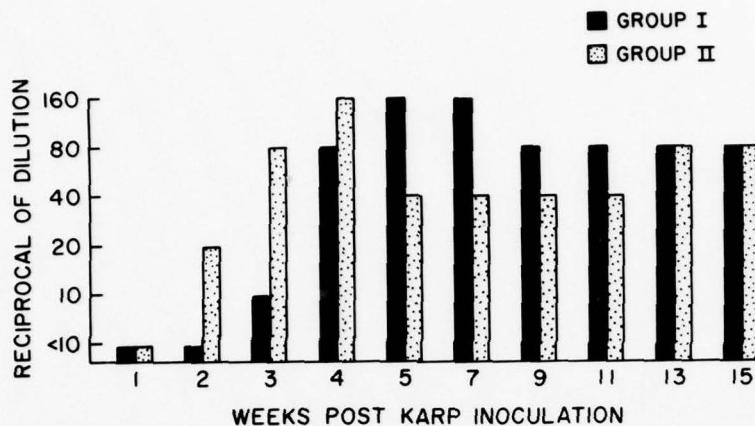


Figure 17. The effect of chloramphenicol regimen on the development of complement-fixing antibodies by mice infected with the Karp strain of R. tsutsugamushi.

treatment), and subsequently at 2 day intervals. Three days after drug withdrawal, 80% of the potential donors withstood secondary Karp challenge, but their spleen lymphocytes were unable to transfer protection. By day 19, the immune protection observed in donors was transferable with spleen cells and a substantial level of protection was observed in recipients for the remainder of the experiment, which was concluded 28 days post infection. By contrast, Group II mice withdrawn from chloramphenicol 10 days after infection, with only 3 days of drug treatment, evidenced complete protection against Karp with development of cell transferable resistance emerging shortly thereafter. Thus, use of the treatment regimen in which proliferation of rickettsiae initially was unaffected by antibiotic resulted in early development of immunity and rapid attainment of the ability to transfer protection with splenic lymphocytes.

It was clear from this study that the efficacy of chloramphenicol treatment for primary Karp infection of mice depended in great part on the time of initiation of treatment. The mice in Group I were treated with chloramphenicol prior to infection which inhibited replication of the rickettsiae until withdrawal of the drug. A substantial number of these mice succumbed to the initial infection if withdrawn from chemotherapy prior to 14 days of treatment, at which time they were immune to rechallenge with Karp. After completion of drug treatment rickettsial proliferation commenced, as indicated by the increased infectious burden in the spleen, and shortly thereafter both detectable CF antibodies and cell-transferable immunity were observed. On the other hand, rickettsial proliferation was allowed to proceed for 7 days in the Group II mice before initiation of chemotherapy. The rickettsial infectious burden in the spleen was high when chemotherapy was initiated, but decreased continuously through

Table 2. Effect of chloramphenicol on the ability of Karp infected mice to withstand rechallenge or to passively transfer protection with spleen cells.

Group	Days after Karp infection <sup>a</sup>	Days after completion/initiation of chloramphenicol treatment	Percent survivors		
			Potential donors after challenge <sup>a,b</sup>	Spleen cell recipients challenged with Karp <sup>b</sup>	
I	17	3 (C) <sup>d</sup>	80	0	
	19	5 (C)	80	80	
	21	7 (C)	100	100	
	23	9 (C)	100	80	
	25	11 (C)	100	100	
	28	14 (C)	100	100	
II	10	3 (I)	100	0	
	11	4 (I)	100	80	
	12	5 (I)	100	80	
	16	9 (I)	100	100	
	24	3 (C)	100	100	
	28	7 (C)	100	100	

<sup>a</sup> Infection and challenge doses consisted of 1,000 MLD<sub>50</sub> of the Karp strain.

<sup>b</sup> Potential donor group for each time point consisted of 10 mice infected with Karp and protected with chloramphenicol. Five mice were challenged with Karp while the remaining five were sacrificed to serve as spleen cell donors.

<sup>c</sup> These animals received 30 X 10<sup>6</sup> lymphocytes from the indicated donor group, followed by Karp challenge 8 hrs later.

<sup>d</sup> C = days after completion of drug treatment; I = days after initiation of drug treatment.

the 28 day observation period. These mice could be removed from therapy after a very short course with little recrudescence of lethal infection. This regimen also fostered more rapid production of serum antibody and cell-transferable protection, both of which became evident approximately one week prior to their appearance in Group I mice.

Several investigators have demonstrated the persistence of rickettsiae in the tissues for long periods after successful treatment. An unexpected development of this study was finding large numbers of virulent rickettsiae associated with non-adherent, lymphocyte enriched, spleen cells. When these cells were briefly homogenized to disrupt their physical integrity and physiological capabilities prior to transfer, fully lethal rickettsiae were easily demonstrated. Yet, the transfer of similar intact cells did not cause death of recipients, but conferred protection on an immunologically naive host against subsequent exogenous challenge. Studies are currently in progress to identify the infected cell population and determine the effect of such parasitism on the development of the immune response.

There appears to be a delicate balance between immunization and proliferation. The marked difference in effectiveness of the Group II treatment schedule suggests that immunization is dependent in part on rickettsial proliferation. A part of this dependence may be due to the accumulation of a critical antigenic mass. Indeed, the necessity for drug-free proliferation was suggested by previous experimental infections in mice and studies in human scrub typhus, in which a short delay in initiation of antibiotic therapy after onset of symptoms resulted in protective immunity with fewer relapses. The temporary rickettsiostasis established with chloramphenicol seemed to preserve the balance between proliferation and immunization, and supported previous data from this laboratory which indicated that animals succumbing to Karp infection mount an immune response which is simply unable to keep pace with intracellular proliferation.

#### B. Host defenses in experimental scrub typhus: Role of spleen and peritoneal exudate lymphocytes in cellular immunity.

In a recent report, we assessed the relative contributions of cellular and humoral immunity in experimental scrub typhus infection of mice by employing strains of Rickettsia tsutsugamushi differing in virulence for mice. Infection with the less virulent Gilliam strain resulted in heterologous protection against an otherwise lethal challenge of the virulent Karp strain. Further, it was shown that significant protection was afforded by the passive transfer of spleen cells and that the protective lymphoid cell in splenic transferates was a thymus dependent lymphocyte.

During these transfer studies, both the immune cells and challenge infection were given intraperitoneally (i.p.). Consequently, the study established only that the spleen contained antigen reactive

cells capable of conferring protection and did not provide information as to whether those spleen cells were in fact the proximate or direct mediators of cellular immunity in the physiologic situation. Exhaustive studies by McGregor and his colleagues (2,3,4,5) have indicated that peritoneal exudate lymphocytes are the physiologic mediator of cellular immunity in other bacterial diseases. These studies led us to explore the possibility that peritoneal exudate lymphocytes are also the physiologic mediators in mice responsible for immunity to heterologous scrub typhus infection. Specifically, we examine the protection elicited against heterologous rickettsial challenge by both spleen and peritoneal exudate lymphocytes administered either intraperitoneally or intravenously; assess the temporal resistance to gamma radiation of lymphocytes obtained from both spleen and peritoneum; and finally, determine the effect of peritoneal exudate induction on the protective capacity of lymphocytes resident in the spleen.

The contribution of various lymphoid cell populations to host protection was evaluated by a series of cell transfer experiments in which the following protocol was followed: BALB/c mice were immunized by a single i.p. inoculation of 100 MID<sub>50</sub> of the Gilliam strain of R. tsutsugamushi. As reported previously<sup>50</sup>, animals tolerated this procedure well and few mice died. At various days after inoculation, five to fifty animals were sacrificed, their spleens and/or peritoneal exudate cells were removed aseptically and appropriate single cell, lymphocyte rich, suspensions were prepared. The cell concentration was adjusted to  $30 \times 10^6$  lymphocytes/0.2 ml, since i.p. inoculation of this number of splenic lymphocytes had previously been shown to provide complete protection against i.p. challenge. Cells were injected either i.p. or i.v. as required by the experiment and recipient animals were challenged i.p. 8 hrs later with 1,000 MLD<sub>50</sub> of the Karp strain. The results were expressed as the percent of ultimate survivors in each group of 10 or more animals.

In general, the protection afforded by transfer of splenic lymphocytes (SpLs) was quite similar to that previously reported and is summarized in Figure 18. Not until 7 days after Gilliam immunization were SpLs able to transfer partial (80%) protection against Karp challenge. Further, all of these survivors evidenced some form of illness such as lethargy and ruffling of fur. Complete protection against both illness and death was achieved with transferates consisting of 14 to 28 day immune SpLs. This protective capacity of immune SpLs declined slightly on days 35 and 42 when experiments were terminated.

The protection afforded by the transfer of peritoneal exudate lymphocytes (PELs) differed from that achieved with SpLs (Fig. 18). Partial protection (20%) from mortality, but not morbidity, was achieved with transfers of PELs as early as day 3. Complete protection against mortality and morbidity was achieved with PELs obtained from animals 7 days after immunization. In addition, this protective

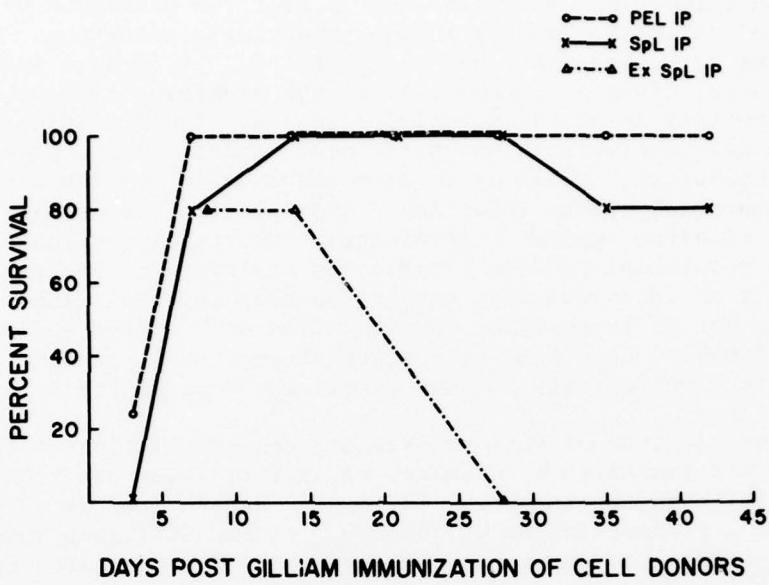


Figure 18. Comparison of the protective effects of Gilliam immune PELs and SpLs transferred by the intraperitoneal route prior to Karp challenge of recipients. In some experiments, immune SpLs were harvested from donors bearing a mineral oil induced peritoneal exudate. Ordinate indicates percent survival of recipients.

capacity of PELs persisted until termination of the experiment and did not show the decline exhibited by SpLs at 35 and 42 days post immunization. However, at these later times (35 and 42 days) all challenged animals exhibited transient signs of illness.

Also summarized in Fig. 18 is the protection afforded by immune SpLs obtained from animals bearing a mineral oil induced peritoneal exudate. The net effect of such an exudate was to reduce the effectiveness of SpL transferates. Complete protection was never achieved and the limited protective capacity dropped rapidly to non-protective levels by 28 days.

In contrast to an i.p. transfer, immune SpLs given i.v. were much less effective at early times in protecting against lethal challenge (Fig. 19). Thus, while i.p. transfer of 7 day immune SpLs achieved 80% protection (Fig. 18), a comparable i.v. transfer of these cells resulted in only 20% protection (Fig. 19). At 14 days post immunization, total protection could be achieved by the transfer of immune SpLs regardless of the route of transfer (Figs. 18, 19).

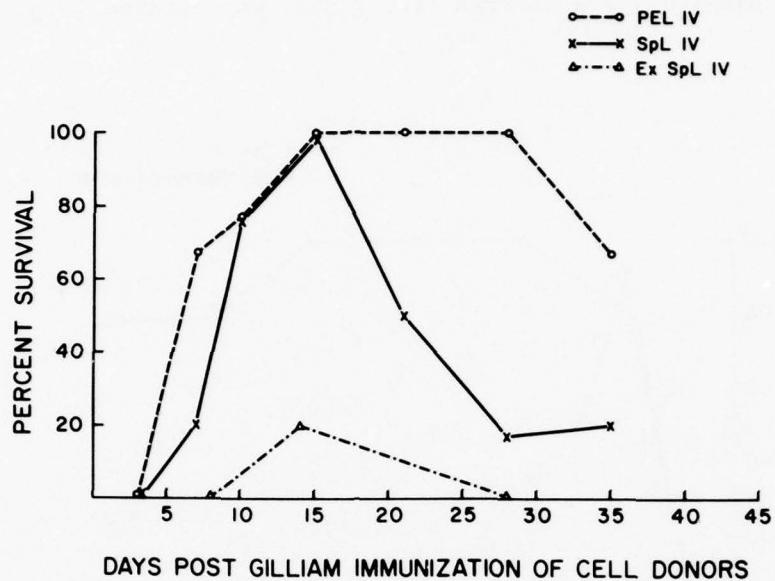


Figure 19. Comparison of the protective effects of Gilliam immune PELs and SpLs transferred by the intravenous route prior to Karp challenge of recipients.

However, while the protection afforded by i.p. transfers of immune SpLs plateaued at these high levels for a few weeks (Fig. 18), the protection afforded by SpLs given i.v. rapidly declined to low levels after day 14 (Fig. 19).

The weak protective response elicited by the i.v. transfer of immune SpLs could be further reduced by the induction of a mineral oil exudate in the peritoneal cavity of donor mice. In this instance, partial protection could only be shown by i.v. transfer of 14 day immune SpLs (Fig. 19), and surviving mice all showed signs of illness.

There were differences in protection between i.v. and i.p. PEL transfers. However, these were less striking than the comparable experiments with SpLs. The complete protection seen with i.p. transfer of PELs at 7 days (Fig. 18) was delayed one week when transfers of similar immune lymphocytes were given i.v. (Fig. 19). Complete protection, once achieved at day 14, plateaued until 28 days (Fig. 19). Unlike i.p. protection, which persisted until day 42 (Fig. 18), i.v. protection declined at day 35, at which time the experiment was terminated (Fig. 19).

The effect of in vitro irradiation (1200 rads) on the protection offered by passively transferred (i.p.) SpLs was studied (Fig. 20).

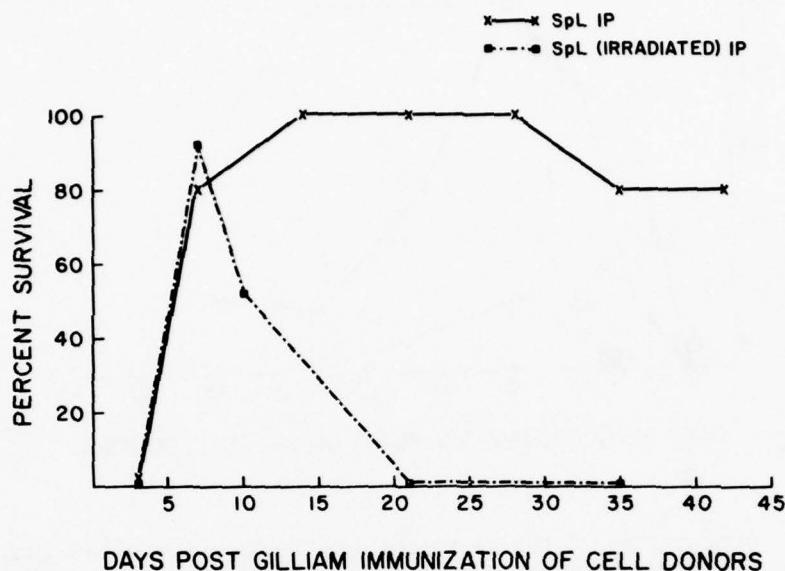


Figure 20. Effect of gamma radiation (1200 rads) given in vitro to Gilliam immune SpLs prior to transfer (i.p.) to recipients subsequently challenged with Karp.

In this experiment the protective effects of  $30 \times 10^6$  untreated or in vitro irradiated immune SpLs are compared. Early in the course of immunization (i.e., at 7 and 10 days), relatively radiation resistant populations of SpLs affording 90% and 50% survival, respectively, were demonstrated. At all other times, exposure to gamma radiation in vitro prior to transfer abolished the protective capacity of immune SpLs. It was of interest that the peak of radiation resistant protection occurred before non-irradiated SpLs (given i.p.) were able to afford complete protection from mortality and morbidity (Fig. 20).

A similar early (8-14 day) radiation resistant population was seen with gamma-irradiated PELs (Fig. 21). In contrast to the SpLs, this radiation resistance occurred after the time at which unirradiated PELs were able to afford complete protection.

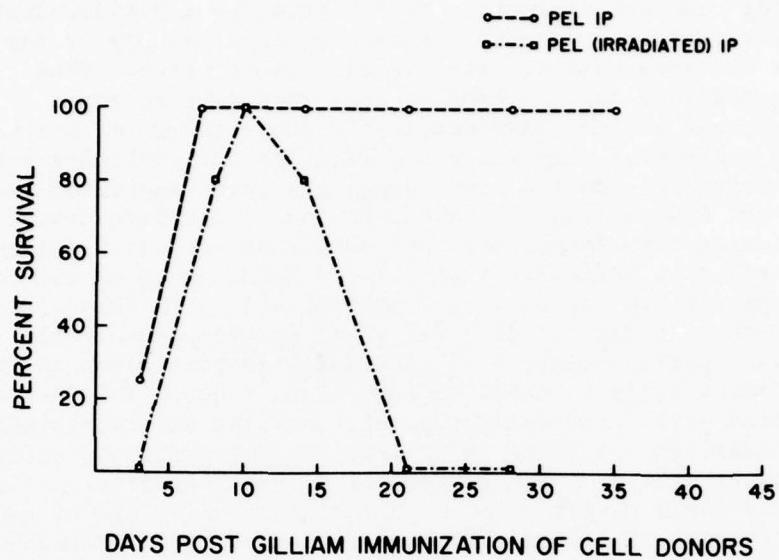


Figure 21. Effect of gamma radiation (1200 rads) given in vitro to Gilliam immune PELs prior to transfer (i.p.) to recipients subsequently challenged with Karp.

Previous experiments from this laboratory have shown that immune spleens contain cells capable of conferring protection when given i.p. and followed with an i.p. lethal infectious challenge of scrub typhus rickettsiae. It was not clear whether SpLs participated in the immune response in vivo, particularly in view of histologic evidence implicating PELs as the mediator of cellular protection against scrub typhus rickettsiae and the considerable data supporting the role of PELs as the mediator of cellular immunity in other bacterial infections.

This study suggests that while SpL transfer represents a valid assay for cell mediated immunity against R. tsutsugamushi, PELs are more likely to represent the physiologic protective cell population. It was clear that PELs represented a more efficient source of immune cells for the following reasons: a) partial protection appeared 4 days earlier and complete protection a week earlier than with SpLs following i.p. transfer; and b) the duration of complete protection after i.p. transfer lasted at least two weeks longer than observed with SpLs. However, these observations could be explained by quantitative differences in immunocompetent lymphocytes. Evidence of qualitative differences was clearly demonstrated in i.v. cell

transfer experiments. While PELs administered by this route were able to confer complete protection over a three week period, similarly transferred SpLs evidenced complete protection only on day 14. Further evidence of a qualitative difference between PELs and SpLs was the fact that protective SpLs resistant to gamma radiation appeared earlier than comparable resistant PELs, but were incapable of conferring complete protection against challenge with virulent rickettsiae. On the other hand, the later appearing radiation resistant PELs evidenced a short period of complete protection. These irradiation experiments were not comparable to the vinblastine studies described by Lefford, *et al* (4) and McGregor *et al* (5) concerning the protective capacity of lymphoid cells. In these latter experiments, the antimitotic drug was given *in vivo* and clearly affected such important aspects of cell mediated protection as the ability of immune cells to enter an infectious focus. The fact that we administered irradiated cells i.p. circumvented any impairment in cellular circulation. A possible explanation for radiation resistance is the existence at that point in time of a large number of antigen reactive cells which obviated the need for division or clonal expansion. Other explanations, including radiation resistant small lymphocytes capable of recruiting the host's immune response, must also be considered. Our data are insufficient for further speculation on the nature of this phenomenon and the experiments were designed solely to compare the functional properties of the two populations.

The data presented here also caution against considering either protective PELs or SpLs as being functionally equivalent during all periods of rickettsial infection. Clearly, the splenic population is temporally heterogeneous with respect to ability to transfer protection by the i.v. route, and lymphocytes from both spleen and peritoneal exudate show temporal differences in radiation resistance. Further evidence for heterogeneity among immunocompetent lymphocytes was seen in experiments in which SpLs used for i.p. transfer were obtained from animals in which a peritoneal exudate was induced by injection of mineral oil. Early in the course of infection only a slight reduction in the protection transferred by SpLs was noted, but at later times (28 days) the induction of such an exudate totally abolished the protective capacity of SpLs. Even more striking results were observed with i.v. transfer, since only a brief and weakly protective capacity of SpLs withstood the concomitant peritoneal exudate. These data indicated that a portion of the spleen cells mediating protection were "mobilizable." The mechanism for depletion of lymphocytes from the spleen by a remote exudate is not well understood. Perhaps a portion of the protective cells are mobilized into the exudate itself and therefore are identical with PELs. Alternatively, a phenomenon of negative lymphoid trapping as described by Zatz and Gershon (6) may have been operative in excluding immunocompetent lymphocytes from the spleen.

The function of the protective cells, whether PEL or SpL, was not defined in this study. It may be that these cells represent the immediate effector cells which interact with macrophages or they may function to recruit the host's own immune response. Whatever the mechanism, the data presented here clearly indicate the heterogeneity of such cells and accentuate the need for further studies to more clearly define the physiologic role of immunocompetent lymphocytes in rickettsial infection.

C. The role of macrophages in resistance to scrub typhus infection.

BALB/c mice survive intraperitoneal (i.p.) infection with 100 MID<sub>50</sub> Gilliam strain of R. tsutsugamushi and are then immune to subsequent lethal homologous and heterologous challenge. The histopathological alterations observed during immunizing Gilliam infection indicate only a minimal and transient proliferation of rickettsial organisms in peritoneal macrophages. On the other hand, in lethal primary infection with Karp strain, rickettsiae multiply in peritoneal macrophages until death of the mouse. Thus, the cellular site of rickettsial activity during containment of infection appears to be the macrophage.

In this study, the ability of BALB/c peritoneal macrophages to contain Gilliam infection and the parameters enhancing intracellular degradation of the rickettsiae have been examined in vitro in an effort to increase ability to monitor and control the sequence of events leading to the immune state. Two methods have been chosen to monitor these events: direct microscopic observation of Giemsa-stained cells and detection of changes in metabolic activity as indicated by incorporation of <sup>3</sup>H thymidine and <sup>3</sup>H adenine. As thymidine is not incorporated by rickettsiae, it can be used to detect the effects of proliferating rickettsiae in the macrophage. On the other hand, adenine incorporation by infected cells is influenced by both the requirements of the rickettsiae and their macrophage hosts. Therefore, the incorporation studies include two controls: uninfected macrophages to monitor the ability of the cells to withstand prolonged culture and macrophages sham-infected with L929 cells that had been subjected to the same harvesting regimen used to produce the Gilliam suspensions. The latter control indicated the response to the tissue culture debris present in the infecting dose.

To determine if peritoneal macrophages from a resistant strain of mice are capable of being infected, adherent peritoneal cells from BALB/c mice were infected in vitro with Gilliam. Figure 22 shows the radioactive adenine incorporation of infected cells and controls. Infected macrophage samples showed increasing incorporation of adenine, while the tissue culture control macrophages returned to normal macrophage adenine levels by day 3 post infection. Although

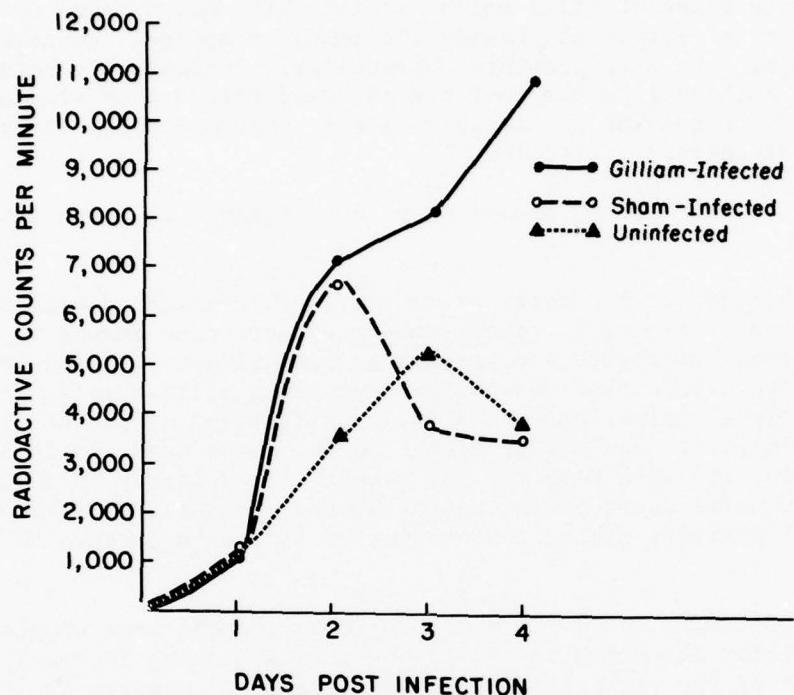


Figure 22. Effect of Gilliam infection on  $^{3}\text{H}$  adenine incorporation by normal BALB/c macrophages.

not indicated in the graph, the thymidine uptake of infected cells was less than that found in normal macrophages. The difference in incorporation was slight, but consistent throughout the studies. Examination of Giemsa-stained cells revealed that 30% of the macrophages were infected initially, and the infection increased to 65% by day 4 post infection. Rickettsiae proliferated exponentially within the infected cells. These results indicate that unprimed macrophages from a resistant strain of mice do not have an inherent capacity to contain scrub typhus infection.

In other studies performed in this laboratory, macrophages from peritoneal scrapings of mice nine days after sublethal Gilliam infection were observed to be highly vacuolated and free of rickettsiae. To test the hypothesis that these macrophages were specifically activated and, therefore, would be resistant to infection, BALB/c mice were immunized by subcutaneous (s.c.) infection with 1,000 PFU Gilliam.

Peritoneal cells were harvested nine days thereafter and the macrophage rich adherent cell populations were infected with Gilliam in vitro. Adenine incorporation curves obtained for the test and the two control populations were similar, peaking on day two and dropping to identical levels on days 3 and 4 post infection. Because the macrophages were removed when the mice were actively infected, background levels of synthetic activity were considerably higher than those seen in unprimed macrophage cultures. The incorporation rates in the test population indicated that few of the macrophages became actively infected. Observations of stained cells were in agreement: 5% of the cells were initially infected and the infection rate remained the same throughout the assay period. However, the cells that were infected appeared to support uninhibited rickettsial growth. Control macrophages were spread, highly vacuolated and free from rickettsiae, indicating that use of s.c. infection for immunization had minimized levels of rickettsiae in the peritoneal macrophages at time of harvest. On the other hand, macrophages taken from mice 18 days post infection presented a totally different picture: 30% of these cells contained rickettsiae when removed from the animal and all cells were in such poor condition that infection in vitro was impossible. As mice normally do survive s.c. infection with 1,000 MLD<sub>50</sub> R. tsutsugamushi and are immune to i.p. challenge by day 28, these results, which indicate that the immune macrophage is not inherently resistant to infection, suggest that, if the macrophage is the effector cell, it must be influenced by other factors generated in the immune response.

The effect of stimulation by activated lymphocyte products on ability of macrophages to resist infection was tested in vitro by exposing normal BALB/c macrophages to supernatant fluids from overnight cultures of lymphocytes that had been obtained 21 days post Gilliam infection. One set of macrophages was incubated with the fluids 4 hours prior to infection and maintained in contact with them throughout the observation period, a second set received lymphocyte supernatants only after infection, and a third set was infected in the normal manner in the absence of lymphocyte supernatant fluids. The results (Fig. 23) do indicate that activated lymphocyte supernatants increased resistance of normal macrophages to infection. Although 10% macrophages preincubated with the lymphocyte culture fluids were infected initially, this was the lowest value, and the highest rate of infection was seen in the unstimulated macrophage contact.

Incubation of rickettsiae with immune serum has been reported to prevent infection of human monocytes by R. mooseri. To determine if antibody played a role in preventing infection of mouse peritoneal macrophages by R. tsutsugamushi, samples of Gilliam were incubated with either normal or immune BALB/c mouse serum prior to infection of unprimed macrophages. Figure 24 illustrates the adenine incorporation of infected and control cells. Cells infected with Gilliam pre-incubated with normal serum consistently incorporated more adenine than control cells, while cells infected with Gilliam pre-incubated

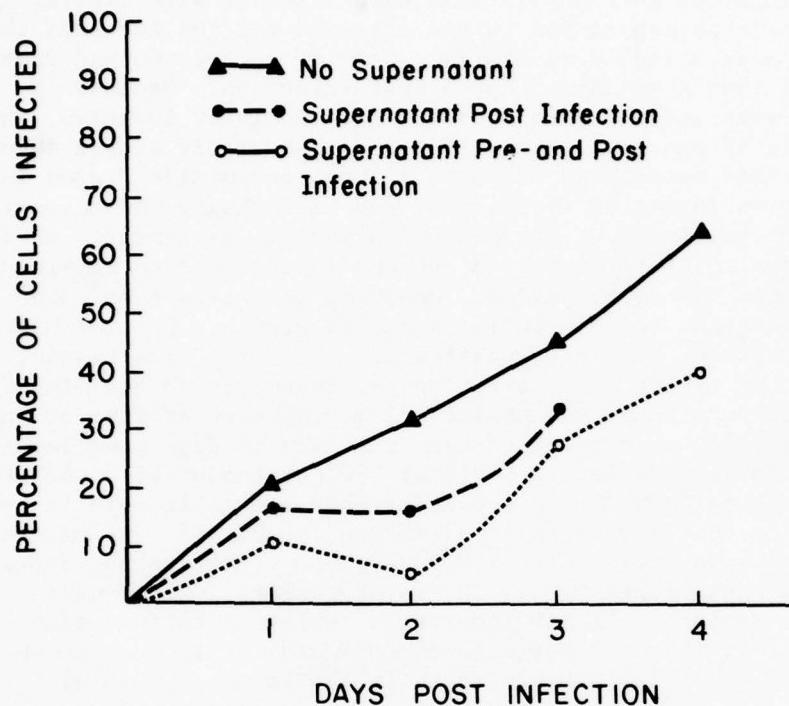


Figure 23. Effect of incubation with infected lymphocyte-activated culture supernatants on the ability of normal BALB/c macrophages to withstand Gilliam infection.

with immune serum had an incorporation profile similar to control cells. The erratic incorporation pattern of this experiment can be attributed to the concentration of serum applied to the cells at the time of infection *in vitro*. Figure 25 illustrates the effect of differing dilutions of normal mouse serum on adenine incorporation by macrophages. The dilution of normal and immune serum used in this experiment was 1:4 and it appears that the minimum dilution of serum that allows approximately linear incorporation of adenine is 1:16. On the other hand, Giemsa staining (Fig. 26) indicated that less than 5% of the macrophages became infected when treated with Gilliam pre-incubated with immune serum, while 25% became infected when treated with Gilliam preincubated with normal serum, the latter value being similar to that obtained by Gilliam alone. This indicates that immune serum may also play a role in prevention of infection, although the mechanism cannot be deduced from these experiments.

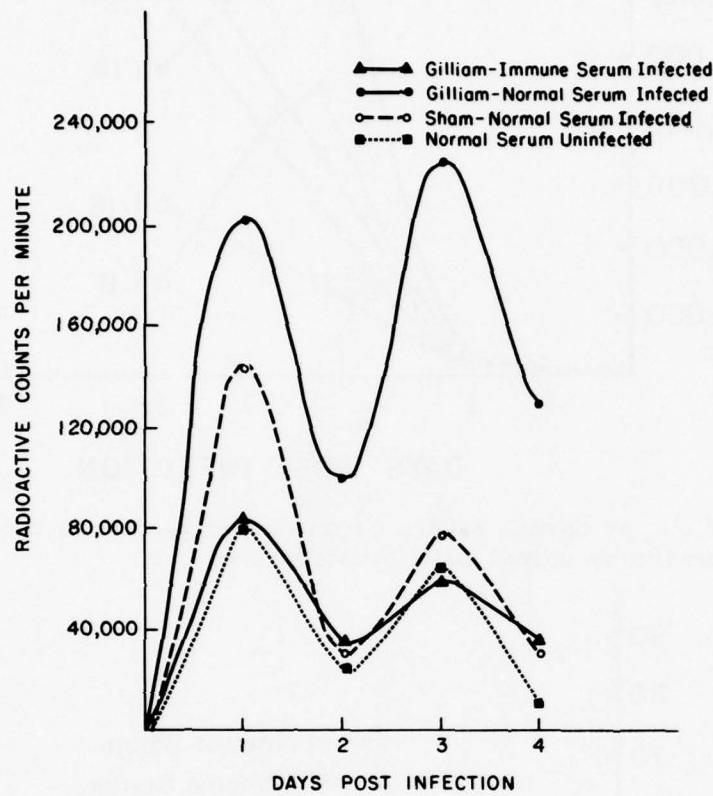


Figure 24. Incorporation of  $^{3}\text{H}$ -adenine by BALB/c macrophages infected with Gilliam that had been preincubated with either normal or immune serum.

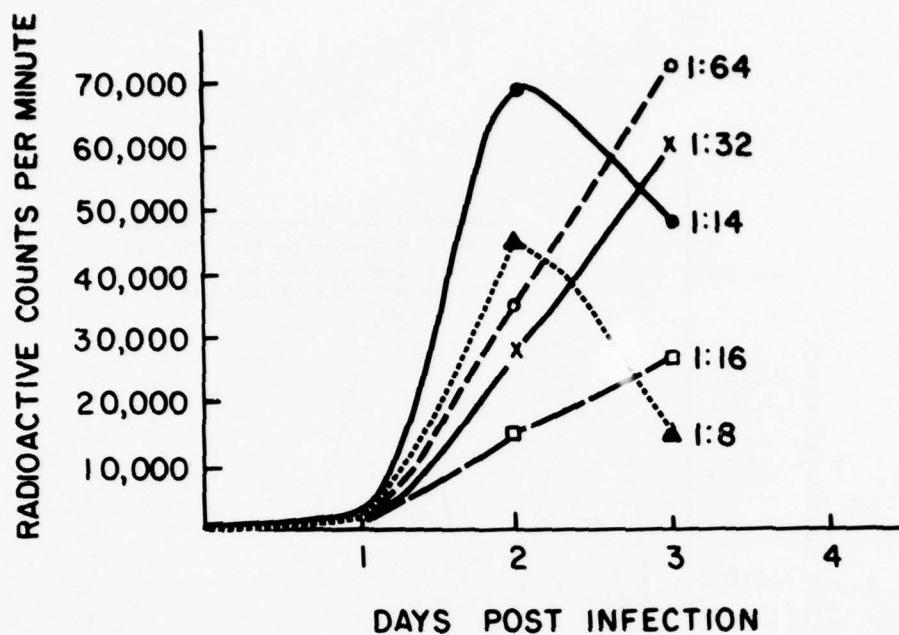


Figure 25. Effect of normal BALB/c serum concentration on incorporation of  $^{3}\text{H}$ -adenine by normal BALB/c macrophages.

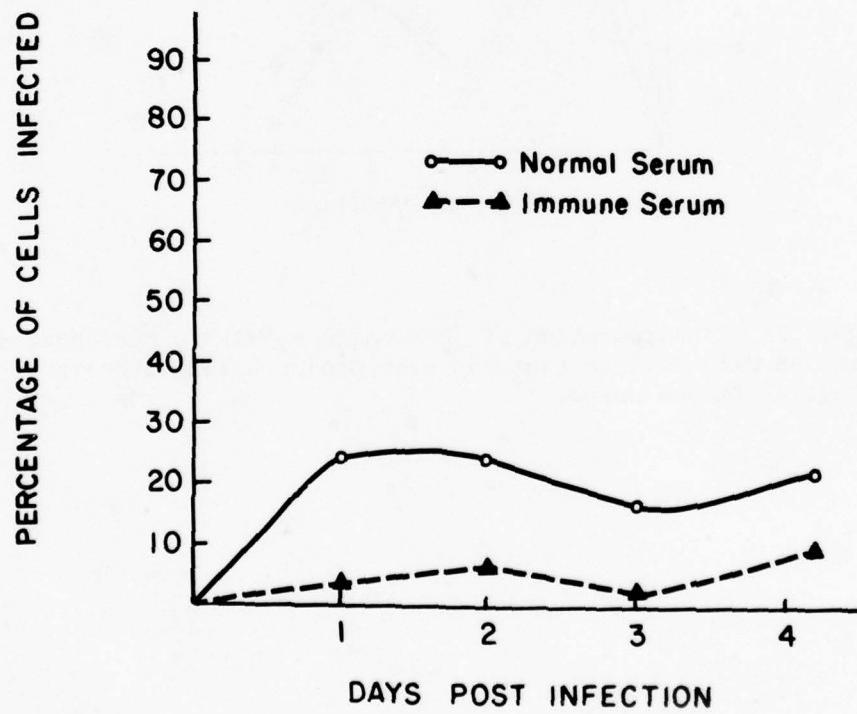


Figure 26. Ability of normal BALB/c macrophages to withstand infection by Gilliam that has been pretreated with either normal or immune BALB/c serum.

Other studies in this laboratory have focused attention on the fact that inbred mouse strains have differing susceptibility to intra-peritoneal Gilliam infection. As macrophages are thought to be the site of rickettsial containment in recovery of BALB/c mice, it was of interest to learn if infection of the susceptible C3H strain could be attributed to a defect in their macrophage population. The kinetics of infection of peritoneal cells and macrophages in vivo was followed for 11 days post i.p. inoculation with 1,000 MID<sub>50</sub> Gilliam. Each day Giemsa stained peritoneal cells from 2 BALB/c mice and 2 C3H mice were examined for percentage of infected cells (Fig. 27). The percentage

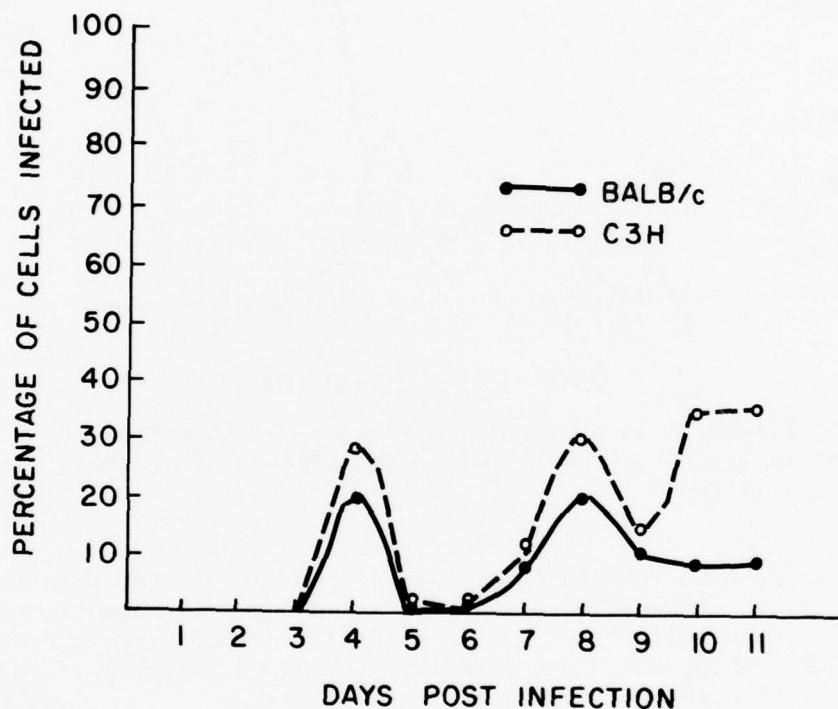


Figure 27. Relationship of mouse strain to the kinetics of infection of peritoneal cells in vivo.

of total infected peritoneal cells in C3H mice was somewhat higher than that seen in BALB/c's, and the difference increased on days 10 and 11, the days of terminal illness in the C3H mice. The percentage of infected cells fluctuated over time in both BALB/c and C3H mice. When the adherent cells were allowed to incubate in vitro overnight, the pattern of infection changed somewhat, as can be seen in Fig. 28.

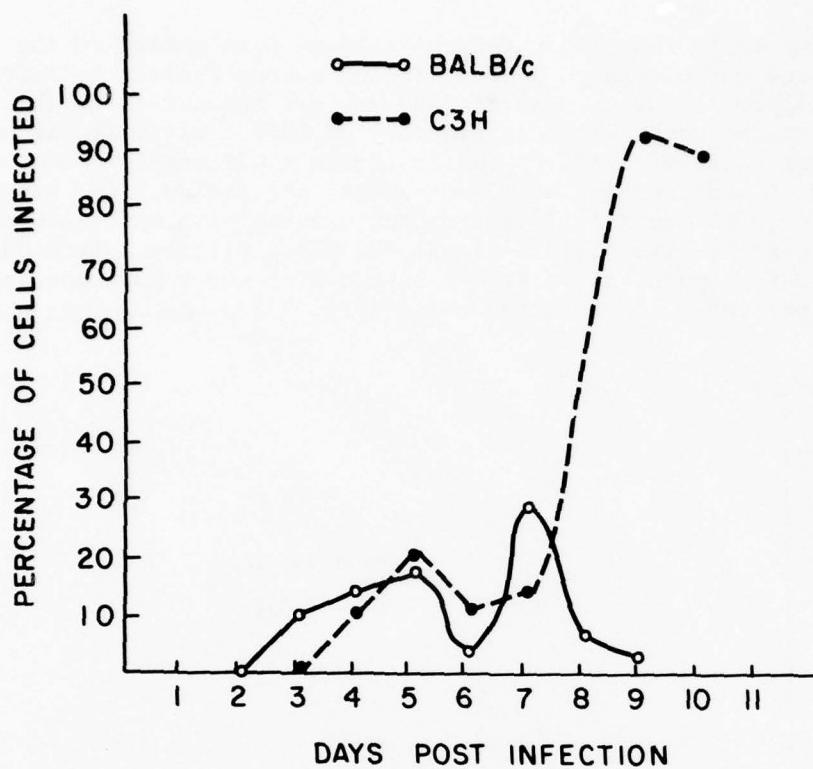


Figure 28. Effect of mouse strain on kinetics of infection of macrophages *in vivo*: percentage of cells infected after overnight incubation *in vitro*.

Rickettsiae appeared in BALB/c macrophages one day earlier than in C3H macrophages. The percentage of C3H macrophages infected increased dramatically on days 8 and 9, a time when BALB/c macrophage infection was only 5%. Again, there was some fluctuation of infected cells in both strains of mice.

As it was possible that macrophages removed from their normal environment and isolated from activated lymphocytes would be prevented thereby from containing an established rickettsial infection, co-cultivation of peritoneal macrophages and lymphocytes from infected BALB/c and C3H mice was attempted. However, a pattern of infection similar to that of macrophages cultured alone was observed. Again, there was a dramatic increase in the number of C3H cells infected on days 8 and 9.

Normal BALB/c macrophages infected *in vitro* sustained an initial infection of 30% of the cells. If there was a defect in the macrophage population of the susceptible C3H mice, infection by the same inoculum of Gilliam could result in an increased number of C3H macrophages sustaining initial infection. Figure 29 shows the adenine incorporation of cultivated normal C3H macrophages infected *in vitro*. The adenine incorporation pattern is similar to that seen with BALB/c macrophages,

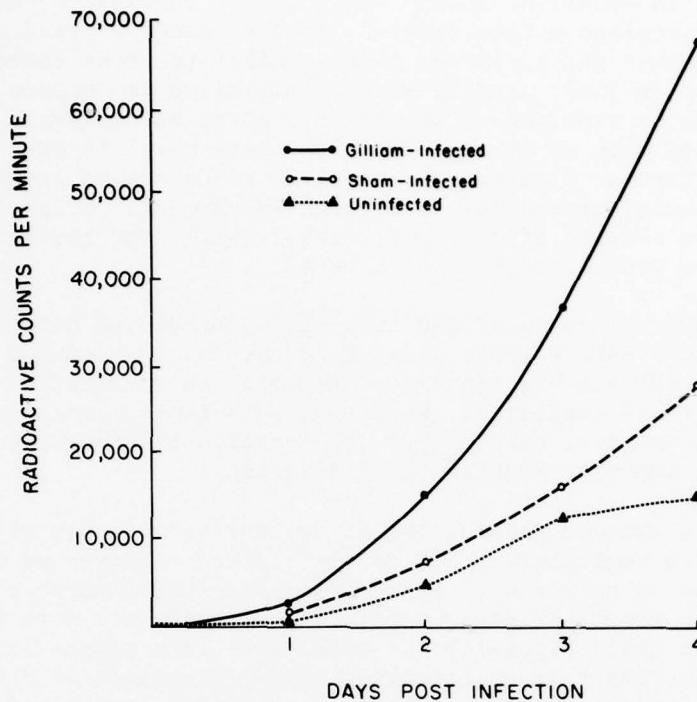


Figure 29. Effect of in vitro Gilliam infection on  $^3\text{H}$ -adenine incorporation by normal C3H macrophages.

(Fig. 22), although the total amount of adenine incorporation was higher in C3H than BALB/c macrophages. The initial peak of adenine incorporation seen in infected and control BALB/c macrophages on day 2 post infection (Fig. 22) was missing from the C3H adenine profile (Fig. 29). This may indicate that C3H macrophages are not as quick to respond to ingested material as BALB/c macrophages, a possibility that will be investigated more intensely in future work.

### III. Development of an inactivated scrub typhus immunogen.

#### A. Development of immunity in mice vaccinated with gamma-irradiated scrub typhus immunogens.

In a recent report we showed that gamma-irradiated scrub typhus immunogens were markedly superior to formalin-killed preparations in protecting vaccinated mice against challenge with homologous and heterologous strains of Rickettsia tsutsugamushi. The majority of

mice vaccinated with the irradiated immunogens were protected against challenge levels in excess of 10,000 MLD<sub>50</sub> of the homologous Karp strain or the heterologous Kato strain. In that study we used vaccination schedules and challenge times similar to those employed by Smadel's group in their studies with formalinized immunogens (7). Although this choice provided an important control mechanism, facilitating comparison of our results with their work, it was recognized that further studies were required to determine optimal conditions for administration of irradiated immunogens. Of particular interest were the effects of antigenic mass, regimen and route of administration on protection levels achieved.

In this study we have used 300 Krad gamma-irradiated Karp immunogens in the inbred BALB/c mouse model to determine the optimum conditions for producing high levels of immunity in vaccinated animals. Using those conditions, we conducted a long term experiment to determine the temporal development and duration of homologous and heterologous immunity induced by vaccination.

To determine whether enhancement of protection with use of multiple-injection regimens was due to the regimen employed or was merely a response to an increase in the total mass of immunogen administered, two groups of mice were vaccinated with the same mass of immunogen, one group receiving the total dose in a single injection of 0.6 ml i.p. and other group receiving three injections of 0.2 ml administered at 5 day intervals. The results of homologous and heterologous challenge are shown in Table 3. Although both procedures elicited high levels of immunity against homologous challenge, immunity indices indicate 100-fold increases in protection against both homologous and heterologous challenge were achieved by delivering the antigenic load in a series of injections over a period of 10 days rather than in a single injection. The differences in protection against heterologous challenge are particularly striking. Although individual mice vaccinated by the single injection regimen were capable of resisting challenge with as many as 100,000 MLD<sub>50</sub> of Kato, the majority of the mice succumbed to challenge at all dilutions tested. On the other hand, 80% of the mice receiving the same antigenic mass delivered in a multiple injection regimen were resistant to challenge with 10,000 MLD<sub>50</sub> of Kato and all were protected against challenge doses of 1,000 MLD<sub>50</sub> or less.

Having established that applying the antigenic mass in a series of injections resulted in greater levels of protection than could be achieved by use of a single injection, it was of interest to determine if further enhancement could be achieved by expanding the time interval between injections. Using the same lot of immunogen, homologous and heterologous protection levels achieved by vaccination using three expanded regimens were contrasted with those achieved with the standard regimen of three injections at 5 days intervals (Fig. 30). No improvement over the standard regimen was achieved by giving the first two

Table 3. Effect of number of injections used to deliver an antigenic mass on survival of mice vaccinated with gamma-irradiated Karp immunogens.

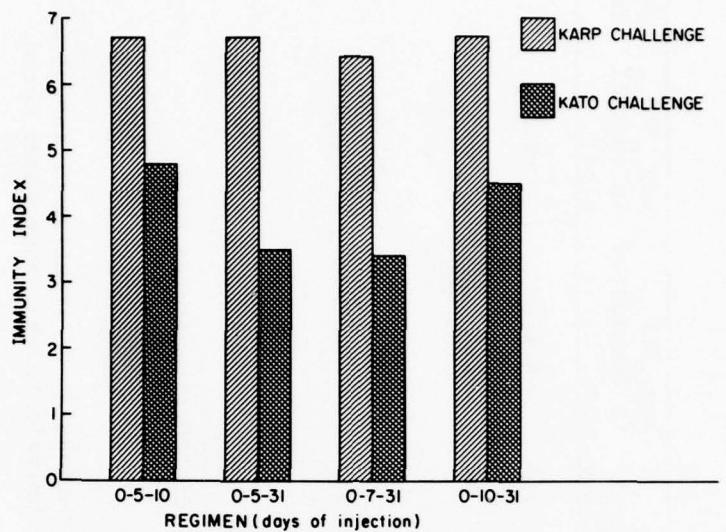
Challenge Strain	No. of immunogen injections <sup>a</sup>	No. survivors/No. vaccinated mice			Log MLD <sub>50</sub> in 10 <sup>0</sup> untrt <sup>b</sup> mice <sup>c</sup>	Log MLD <sub>50</sub> in 10 <sup>0</sup> trt <sup>b</sup> mice <sup>c</sup>	Immunity Index <sup>d</sup>		
		100M <sup>b</sup>	10M	1M					
Karp	1	0/5	0/5	1/5	5/5	5/5	-4.1	-9.5	5.4
	3	0/5	3/5	5/5	5/5	ND	-2.5	-9.5	7.0
Kato	1	ND	0/5	0/5	1/5	1/5	-6.0	-8.0	2.0
	3	ND	0/5	0/5	0/5	4/5	-3.9	-8.0	4.1

<sup>a</sup> Total antigenic mass delivered was  $2.5 \times 10^8$  MLD<sub>50</sub> of irradiated rickettsiae.

<sup>b</sup> Approximate challenge dose (MLD<sub>50</sub>).

<sup>c</sup> Values are based on exact challenge doses, which were determined from titration of inoculum in control mice and dilution factors used to achieve the approximate challenge doses noted.

<sup>d</sup> Immunity index =  $\log_{10}$  MLD<sub>50</sub> in vaccinated mice -  $\log_{10}$  MLD<sub>50</sub> in control mice.



**Figure 30.** Effect of temporal expansion of multiple-injection regimens on protection. Mice were challenged 21 days after completion of each vaccination regimen. All injections were administered intraperitoneally.

injections 5-10 days apart and the final injection at one month. Immunity indices following homologous challenge remained practically constant and, although there is an apparent decrease in heterologous protection with use of the two middle regimens, analysis of the data by the Spiermen-Karber method (8) indicated that the differences were not significant at the 95% level.

The results of a test to determine the levels of protection that could be achieved by subcutaneous (s.c.) vaccination are shown in Table 4. The mice had received three injections of 0.2 ml immunogen at 5 day intervals and were challenged on day 31. Although significant levels of protection were achieved, comparison with challenge results depicted in Table 3 and Fig. 30 reveals that levels of immunity to homologous and heterologous challenges were lower by 100-fold and 10-fold, respectively, than those observed after i.p. vaccination using the same regimen.

Since modifications in regimen and route of inoculation did not result in increased immunity to challenge, the standard regimen of three i.p. injections at 5 day intervals was retained and used in a

Table 4. Protection of mice vaccinated subcutaneously with gamma-irradiated Karp immunogens.

Challenge Strain	No. of survivors/no. of vaccinated mice challenged		Log <sub>10</sub> MLD <sub>50</sub> in vaccinated mice <sup>b</sup>		Log <sub>10</sub> MLD <sub>50</sub> in control mice <sup>b</sup>		Immunity Index <sup>c</sup>
	10M <sup>a</sup>	1M	10K	1K	100	mice	
Karp	0/5	2/5	4/5	5/5	4/4	-4.0	-9.0
						5.0	
Kato	0/5	0/5	1/5	1/5	5/5	1/5	-5.0
						-7.9	2.9

a Approximate challenge dose (MLD<sub>50</sub>).

b Values are based on exact challenge doses, which were determined from titration of inoculum in control mice and dilution factors used to achieve the approximate challenge doses noted.

c Immunity index = log<sub>10</sub> MLD<sub>50</sub> in vaccinated mice - log<sub>10</sub> MLD<sub>50</sub> in control mice.

series of experiments to determine the development and maintenance of immunity following vaccination. The results are depicted in Fig. 31. Immunity to homologous challenge developed quite rapidly, being at maximum level when first tested on day 17, 7 days after the

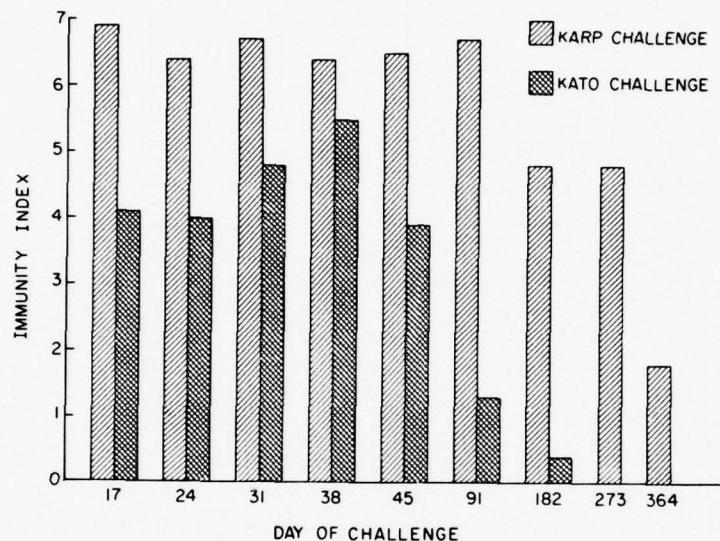


Figure 31. Development and duration of homologous and heterologous immunity achieved by vaccination with gamma-irradiated Karp immunogens. Mice were vaccinated by 3 i.p. injections of 0.2 ml immunogen administered at 5 day intervals and were challenged by i.p. injection.

final injection of immunogen, and remained at that level for 3 months. After that time protection levels slowly decreased, although the mice remained resistant to challenge with greater than 10,000 MLD<sub>50</sub> of Karp at 9 months, and to a 100 MLD<sub>50</sub> challenge at the end of a year, when the experiment was terminated. Immunity to heterologous challenge developed more slowly and waned far more rapidly than homologous immunity. Although protection levels were high on day 17, they did not reach peak levels until day 38. Resistance then began to wane, evidencing a 10-fold decrease at day 45 and becoming unacceptable low by day 91.

In our first report on the effects of vaccination with gamma-irradiated immunogens we noted that protection against homologous challenge was heightened 100-fold by increasing the regimen from one injection of  $10^6$  irradiated rickettsiae to either three injections of  $10^6$  or one injection of  $10^7$  organisms, and that absolute immunity to challenge with 10,000 MLD<sub>50</sub> of Karp was achieved when the mice were given three injections of  $10^7$  irradiated Karp. This increase could have been due to either the temporal regimen employed or merely to accumulation of a larger amount of immunogen in the host. The additional data accumulated in the present study indicate that both factors contribute to enhancement of protection. The use of one injection of  $10^7$  organisms had protected 70% of the vaccinated mice against challenge with 10,000 MLD<sub>50</sub> of Karp, whereas all mice that received an antigenic mass of approximately  $10^8$  rickettsiae in a single injection in this study survived the 10,000 MLD<sub>50</sub> challenge and 80% evidenced resistance to 100,000 MLD<sub>50</sub> Karp. The contribution of temporal regimen can be seen as an additional 40-fold increase in protection when the same total antigenic mass of  $10^8$  organisms was administered in smaller doses over a series of three injections at five day intervals. However, it is interesting that further temporal expansion provided no concomitant increase in protection, as Plotz and co-workers, using formalinized suspensions, reported a 10-fold boost in homologous protection achieved by a similar expansion of their vaccination regimen (9). Of course, their immunity indices prior to booster were in the range of 1.8 to 4.4 as opposed to the 6.4 to 7.0 range of immunity indices achieved with use of gamma-irradiated immunogens. Thus, this lack of booster effect may be only an indication that no immune response is capable of handling challenge doses in excess of one million MLD<sub>50</sub>.

The value of the multiple-injection regimen becomes particularly evident with examination of the response to heterologous challenge. When applied in a single injection, an antigenic mass of  $10^8$  irradiated rickettsiae yielded approximately the same level of protection against Kato challenge as had been reported previously for one injection of  $10^7$  organisms. On the other hand, protection increased 100-fold when this antigenic mass was applied over a series of three injections. As with other systems employing inactivated immunogens, increasing the number of immunogen injections apparently tended to broaden the immune response. Again, however, no increase in protection was observed with expansion of the time interval between injections.

Although vaccination by the subcutaneous route did not appear to be as efficacious as i.p. injection, the fact that significant levels of protection were achieved serves as further proof of the overall superiority of gamma-irradiated scrub typhus immunogens to their formalinized predecessors, which other investigators have reported to be almost totally ineffective when administered subcutaneously.

The response to i.p. vaccination was rapid. Homologous immunity had reached peak level within 7 days of completion of the vaccination regimen, remained unchanged for at least 3 months, and was effective against challenge with greater than 10,000 MLD<sub>50</sub>. Karp when tested at 9 months. By 12 months, however, the animals were found only to be resistant to challenge with fewer than 100 MLD<sub>50</sub> Karp. Thus, initial levels of homologous immunity are higher, and remained higher for 9 months, than initial levels reported for mice vaccinated with formalinized immunogens. On the other hand, immunity observed after recovery from experimental infections is longer than that achieved in this study: Fox (10) found that mice surviving infection remained infected, but immune to a 10,000 MLD<sub>50</sub> homologous challenge until the termination of his experiment at 610 days. Unfortunately, no similar animal studies are available for comparison with our heterologous challenge results. However, our results do compare favorably with observations from previously reported studies on experimental infection of humans, who were found to be resistant to homologous challenge for up to a year while susceptible to heterologous infection in as short a period of time as one month. Nevertheless, although gamma-irradiated immunogens prepared from the Karp strain of R. tsutsugamushi are far more immunogenic than previously studied inactivated suspensions, their inability to induce long-lasting heterologous immunity remains a serious deficiency.

We are now exploring use of other strains as immunogens and administration of multiple-strain immunogens, either simultaneously or in successive injections, in an effort to increase the levels of protection and duration of immunity to heterologous challenge. Further, the discovery that C3H mice are susceptible to challenge with many strains of R. tsutsugamushi to which BALB/c mice are resistant will allow us to increase our knowledge on the breadth of protection that can be achieved against heterologous challenge.

B. Examination of inactivated rickettsial suspensions for the presence of replicating organisms.

In the annual report for FY 76, we compared levels of homologous and heterologous protection achieved in mice vaccinated with either gamma-irradiated or formalinized scrub typhus immunogens. However, it was recognized that the inactivation procedures employed, particularly gamma irradiation, could have resulted in the survival of a small number of resistant organisms that did not express their lethal potential when inoculated into mice because the vastly greater number of inactivated organisms initiated an immune response capable of suppressing proliferation. Therefore, two in vitro techniques were employed to search for replicating organisms in the immunogen preparations. The growth of untreated, gamma-irradiated, and formalinized rickettsiae in L-929 cells is shown in Table 5. Both untreated and irradiated organisms rapidly entered the cytoplasm of cells, but uptake of formalinized rickettsiae was not observed. Untreated rickettsiae

Table 5. Growth of untreated and inactivated Karp in irradiated L-929 cells.

Treatment	Severity of cell infection <sup>a</sup>	% Cells infected				
		0 <sup>b</sup>	1	3	5	7
Untreated	H	0	0	3	12	30
	M	34	31	44	50	40
	L	62	60	30	11	13
	O	4	9	23	27	17
Gamma radiation (300 Krads)	H	0	0	0	0	0
	M	0	0	0	0	0
	L	27	19	6	3	2
	O	73	81	94	97	98
Formalin	H	0	- <sup>c</sup>	-	-	-
	M	0	-	-	-	-
	L	0	-	-	-	-
	O	100	-	-	-	-

<sup>a</sup> Evaluation of infected cells was made on the following basis: Heavy infection (H), > 20 rickettsiae/cell; moderate infection (M), 6 to 20 rickettsiae/cell; light infection (L), 1 to 5 rickettsiae/cell; and uninfected (O), no visible rickettsiae.

<sup>b</sup> Day postinfection

<sup>c</sup> -, Not done

continued to multiply throughout the incubation period, but there was no indication of replication by the irradiated Karp. There was, apparently, gradual elimination of irradiated intracellular organisms, since the percentage of cells evidencing a light infection decreased during the observation period. This technique allowed detailed observation of the fate of a representative inoculum of gamma-irradiated rickettsiae, but the possibility remained that the sample was not large enough to contain one of a small number of viable organisms. The plaque assay was more suitable for screening large numbers of rickettsiae for radiation-resistant survivors. Portions of a Karp suspension having a titer of  $4.8 \times 10^8$  MLD<sub>50</sub>/g of yolk sac were assayed before and after irradiation with 300 Krads. The pre-exposure titer was  $2.7 \times 10^8$  plaque-forming units/g of yolk sac. After irradiation, the Karp suspension was diluted 1.2, and each of 32 plates was seeded with 0.1 ml of inoculum. This represented a total of 0.32 g of infected yolk sac containing  $1.5 \times 10^8$  potential MLD<sub>50</sub>, a number that exceeds the amount of rickettsiae contained in one injection of irradiated immunogen. No plaques were observed on any plate.

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Work Unit 006 Rickettsial Diseases of Military Personnel

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Presentations:

1. Eisenberg, G.H.G., Jr. and Osterman, J.V. Development of immunity in mice vaccinated with gamma-irradiated scrub typhus immunogens. *Annual Meeting of the American Society for Microbiology*, New Orleans, La. 1977 (May).
2. Ewing, E.P., Jr., Takeuchi, A., Shirai, A. and Osterman, J.V. Electron microscopic studies in peritoneal mesothelium of BALB/c mice infected with scrub typhus rickettsiae. *Annual Meeting of the American Society for Microbiology*, New Orleans, La. 1977 (May).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>1</sup> DA OB 6526	2. DATE OF SUMMARY <sup>2</sup> 77 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCRTY <sup>3</sup> U	6. WORK SECURITY <sup>4</sup> U	7. REGRADING <sup>5</sup> NA	8. DISB'R INSTN'N NL	9. SPECIFIC DATA - <input checked="" type="checkbox"/> CONTRACTOR ACCESS <input type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: <sup>6</sup> PROGRAM ELEMENT 62770A	PROJECT NUMBER 3M762770A802			TASK AREA NUMBER 00	WORK UNIT NUMBER 007		
11. CONTRIBUTING XEROX CARDS 114F							
11. TITLE (Provide with Security Classification Code) <sup>7</sup> (U) Field Studies of Rickettsioses and Other Tropical Diseases							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>8</sup> 010100 Microbiology 002600 Biology							
13. START DATE 73 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House				
17. CONTRACT/GRAANT NA	18. RESOURCES ESTIMATE EXPIRATION: FISCAL YEAR 77 CURRENT 78	19. PROFESSIONAL MAN YRS 5 5	20. FUNDS (In thousands) 88 30				
21. DATES/EFFECTIVE: D. NUMBER: E. TYPE: F. KIND OF AWARD:	G. AMOUNT: F. CUM. AMT.						
22. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research	23. PERFORMING ORGANIZATION NAME: US Army Medical Research Unit Malaysia						
ADDRESS: Washington, DC 20012	ADDRESS: Kuala Lumpur, Malaysia						
RESPONSIBLE INDIVIDUAL NAME: RAPMUND, Garrison, COL TELEPHONE: 202-576-3551	PRINCIPAL INVESTIGATOR (Punish SEAN if U.S. Academic Institution) NAME: Huxsoll, D. L., LTC, VC TELEPHONE: SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: NAME:						
24. GENERAL USE Foreign intelligence not considered							
25. KEYWORDS (Provide EACH with Security Classification Code) (U) Scrub typhus; (U) R. tsutsugamushi; (U) Human volunteer; (U) Laboratory diagnosis; (U) Epidemiology; (U) Immunity; (U) Ecology; (U) Leptotrombiculid spp; (U) Ehrlichia canis							
26. TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Punish individual paragraphs identified by number. Precede last of each with Security Classification Code) 23(U) To investigate the incidence of scrub typhus infection in selected local and foreign populations, to relate this to the prevalence of vector chiggers and their common rodent hosts, to determine the strains of R. tsutsugamushi causing human infections and relate to clinical disease, to develop improved serological methods, to evaluate simple and effective means of treatment, to study the basis for immunity, to define vector-rickettsia relationships, to develop a laboratory animal model for the disease, and to produce laboratory animals in support of the mission. Scrub typhus is an incapacitating disease of military importance. 24(U) Provides scientific reagents and equipment which can be best procured in the US for shipment to and use by WRAIR personnel in Malaysia functioning collaboratively with Malaysian scientists supported under USAMRDC grant reported on DA OA 7413. 25(U) 76 10 - 77 09 Studies of febrile illnesses at a rural hospital in Pahang, Peninsular Malaysia, have shown that a definitive diagnosis can be achieved on 60-80% of febrile cases admitted and that scrub typhus remains the most common cause of fever. A single oral dose of 200 mg of doxycycline was shown to be as effective as a seven day course of tetracycline in treating scrub typhus. A direct immunofluorescence test was developed for identification of R. tsutsugamushi organisms in chiggers. The technique has been used to determine distribution of various antigenic strains of R. tsutsugamushi in different habitats and to establish a relationship between infections in chiggers and rodent hosts. A significant reduction in chiggers was observed in an area where an acaricidal-treated rodent bait had been used. Serological studies on sera collected from dogs in selected habitats showed that dogs may be useful sentinel animals for scrub typhus and murine typhus. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.							
* Available to contractors upon originator's approval.							

DD FORM 1498 (MAR 68)

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M762770A802 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 007 Field Studies of Rickettsioses and Other Tropical Diseases

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SINGLE DOSE DOXYCYCLINE THERAPY FOR PRESUMPTIVE SCRUB TYPHUS

Scrub typhus occurs throughout much of the Asian-Pacific region. It was a significant military medical problem during the second world war (84), and also in American troops in Vietnam (22). Serological surveys (17, 83) and studies of febrile patients (69, 11) have shown a very high prevalence of infection among the rural population of Malaysia. Tetracycline eliminates fever and other symptoms more rapidly than chloramphenicol, and relapses are not common unless short courses of either drug are started four days or less after the onset of symptoms (87).

A single dose of 100-200 mg of doxycycline, a long-acting derivative of oxytetracycline, is as effective as a 10 day course of tetracycline or chloramphenicol in the treatment of louse-borne typhus (61). Single dose therapy for scrub typhus would shorten the time patients spend in hospital, and might allow treatment to be given as an outpatient, since consumption of the antibiotic could be assured.

A definite clinical diagnosis of scrub typhus is often difficult to achieve, however, since an eschar and rash are not seen in the majority of Malaysian cases, and common clinical features of scrub typhus, such as fever, headache and cough, do not distinguish it from other infections (66, 11). Likewise, laboratory confirmation is rarely available early enough to influence management.

The study was designed to compare a single dose of doxycycline and a seven day course of tetracycline, in selected subjects at a hospital where over 20% of febrile patients were known to have scrub typhus (11). It had been standard practice to start tetracycline within 48 hours of admission, unless an alternative diagnosis was made, since the high prevalence of scrub typhus had been recognised.

Patients of at least 18 years of age admitted to Mentakab district hospital, Malaysia, between September 1976 and July 1977, for investigation of febrile illness, were recruited to the trial. Patients were excluded if: (a) they had already received tetracycline or chloramphenicol; (b) there was a history of allergy to tetracyclines; (c) they were jaundiced; (d) they were pregnant; (e) there was clinical or laboratory evidence of a non-rickettsial disease, including those with malaria parasites in blood films and those with a Widal 'O' titer of 1/160 or more on admission; (f) they did not consent after reading a fact sheet written in their own language and supplemented by a verbal explanation.

Volunteers were randomly assigned to one of the following treatment groups: (a) doxycycline 200 mg; (b) tetracycline 500 mg six hourly for seven days.

A thick film was examined for malaria parasites on three occasions within 48 hours of admission, after four days, and again if fever persisted after treatment. Hemoglobin concentration, white blood cell and differential counts were performed on admission to hospital.

Throat swab, sputum, mid-stream urine (MSU) and two separate venous blood samples were cultured by standard techniques.

Specimens of serum were collected on admission to hospital, seven to 10 days later, and at 14 days if possible, and kept at -20°C until examined. The sera were examined for antibody to Rickettsia tsutsugamushi by indirect immunofluorescence (IFA); to Leptospira spp by the hemolytic (HL) test; to Pseudomonas pseudomallei by indirect hemagglutination (IHA); to groups A and B arboviruses by hemagglutination-inhibition; and for febrile agglutinins using Salmonella group D (Widal 'O' & 'H'), Proteus OXK and OX19, and Brucella abortus. In all instances in which apparently conflicting results were obtained, titrations were repeated for confirmation.

Isolation and identification of R. tsutsugamushi was performed by mouse inoculation.

Sera collected on admission to hospital were screened for tetracycline and chloramphenicol by a microbiological technique. Five ul of each serum was pipetted onto a sterile disc and the discs placed on agar plates seeded with two organisms (*Escherichia coli*, NCTC 10418; *Staphylococcus aureus*, NCTC 6571). Control antibiotic discs were utilized. Any sera inhibiting growth were retested after the addition of penicillinase.

A diagnosis of scrub typhus was confirmed by one or more of the following criteria:

- (a) isolation of R. tsutsugamushi

(b) a four-fold or greater rise in IFA titer, to at least 1/200, or a static titer of 1/800 or more.

(c) a four-fold or greater rise in OXK titer, to at least 1/160, or a static titer of 1/640 or more, provided that there was no evidence of an alternative diagnosis.

Diagnosis of other infections was by isolation of known pathogens, or by the demonstration of significant rises in antibody titer, or of static high titers in the absence of other diagnoses.

Leptospiral HL and Widal 'O' titers of 1/320 or more were considered significant in the latter case.

Patients were observed for 14 days from start of therapy. They were asked to remain in hospital for seven days, and to return for observation on the 10th and 14th days. Defaulters were visited at their homes. Temperature recordings were made four hourly by the nursing staff, and the patients were seen at least once a day by a doctor while in hospital. Records were kept of the patients' complaints of headache and cough and of the time taken to return to normal. Temperatures of less than 37.2°C were regarded as normal. Similar observations were also made on days 10 and 14.

Any patient who had not shown a marked improvement within 48 hours of starting therapy, or in whom laboratory or clinical evidence of an alternative diagnosis appeared, was given additional treatment at the discretion of the clinician in charge.

The collection of specimens and entry of patients to the trial are now complete. Acquisition of some results is still awaited and, thus, final analysis is not yet possible.

One hundred and forty-nine rural Malaysian patients admitted with fever of unknown origin were randomly assigned to two treatment groups. The response to a single oral dose of 200 mg of doxycycline was compared with that to a seven day course of tetracycline, 500 mg six hourly. One patient was eliminated as not satisfying the trial entry criteria.

Of the remaining 148 sixty-five patients (43.9%) eventually fulfilled the criteria for definite diagnosis of scrub typhus, 35 receiving doxycycline and 30 tetracycline. *R. tsutsugamushi* was isolated from 49 (73.8%) patients. There was no difference between the groups in time to defervescence, abolition of cough and headache, or in the time taken to recover well-being. There were two relapses, one in each group.

A diagnosis of leptospirosis was made in 16 (10.8%) patients, and typhoid or paratyphoid in 12 (8.1%) subjects. Dual infections

were common. There was no difference in response, between the two drugs, though five of those with typhoid required additional therapy. Antibiotic therapy may have been irrelevant in many of the remaining 60 cases, and there was again no difference in response between the two drugs. Group B arbovirus infections were apparent to the extent of approximately 14%.

No serious disadvantage was found to treatment of selected, though undiagnosed, febrile patients with a single dose of doxycycline, provided that appropriate investigations had been initiated, and therapy reconsidered after 48 hours if the response were unsatisfactory.

Because of the complications arising out of the high incidence of apparent multiple infection, the trial will be reported in two linked papers:

1. Single dose doxycycline therapy for presumptive scrub typhus.
2. Problems of diagnosis and therapy of patients with fevers of unknown origin in a rural Malaysian hospital.

#### HOSPITAL BASED PUO STUDY

This study continues to be executed at the District Hospital Mentakab, Pahang. Additional information is obtained from samples forwarded by other hospitals (v.i.):

1. Mentakab: The format of the study remains unchanged. Patients have been admitted to the study at an average rate of approximately 40 monthly. A detailed breakdown of diagnostic categories is not currently possible due to the transfer to ADP format of data recording before the installation of ADP equipment. It is, however, clear that approximately 20% of cases are due to scrub typhus, some 10%+ due to leptospirosis and over 5% due to typhoid. By use of special logistic arrangements, patients admitted to the trial are currently the main source of specimens for CMI and monocyte culture studies.

2. Other Hospitals: The only hospitals contributing numbers of sera remain Seremban and Kuala Pilah, both in Negeri Sembilan. In both cases, the study is bedevilled by uncontrolled selection, personnel changes and lack of standardized information. In the case of Seremban, the picture is further complicated by the hospital being a General Hospital, thus, a point of secondary referral. Nevertheless the processing of samples from these two centers remains a useful monitor on the distribution of causal diagnoses of PUO cases within the general geographic area of the

origin of patients in the main studies. Initial efforts have been made to standardize the acquisition of data and its recording. Detailed breakdown of diagnoses is not currently available but the picture for Kuala Pilah is much like that at Mentakab while at Seremban the proportion of patients in whom a causal diagnosis is reached is much less than the 70% at the District Hospital level.

#### A STUDY OF THE CLINICAL, PATHOLOGICAL, EPIDEMIOLOGICAL AND THERAPEUTIC CHARACTERISTICS OF SCRUB TYPHUS AMONG FEBRILE PATIENTS AT AN ISOLATED RURAL HEALTH CENTER IN MALAYSIA.

The purpose of this study is to establish the current picture of febrile illnesses - with particular emphasis on scrub typhus - among a susceptible population in areas of high risk and at an early stage of the disease. Studies among febrile patients presenting at rural hospitals and other studies at a FELDA settlement have shown FELDA oil palm settlers to be susceptible and at risk. However, the pattern of disease among hospital patients is likely to be very different from that at the level of the rural health center, and it is known that the average length of fever at the time of admission to hospital is 7-8 days. Also at the earlier studies at Bukit Mendi the alternative sources of health care delivery, other than the rural health center, were numerous and readily available, and it is probable that the limited population there is already beyond the susceptible period and (due to ecological changes) is not exposed to the same high level of risk. An area was sought, therefore, that overcame these problems and was identified as the FELDA complex known as the Jengka Triangle in Western Pahang.

In addition to the advantages from the viewpoint of human studies, the complex (which consists of some 23 schemes of nearly 4000 acres each) offers a unique opportunity to study relevant chigger ecology at all stages of development from primary jungle to established plantations and relate this to actual and concurrent human infection. There are sufficient schemes at an early enough stage of development for the complex to be the site for future trials of prophylactic agents, at which time the information gathered in this survey will be invaluable as a basis for predictive of expected infection/disease rates.

In essence the method of study used in the hospital based 'PUO study' is translocated to the rural health center in the middle of the complex, but certain modifications are necessary to accomodate the local and logistic problems inherent due to the relative inaccessibility of the chosen site.

1. Patient Selection: All febrile patients seeking treatment at Jengka Rural Health Center, or discovered by checking

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call absentees at the longitudinal study sites, and who cannot be labelled with a causal diagnosis are admitted to the study. The decision on diagnosis and, thus, admission to the trial is made by the local doctor in clinical charge or in his absence by a Hospital Assistant. Clinical management and decisions remain at all times in the hands of those normally making them - i.e. the local doctor or Hospital Assistant.

2. Initial Procedures: In addition to the clinical examination and other available investigations indicated, all patients will have the following laboratory tests:

a. Samples taken:

- (1) Urine
- (2) Throat swab
- (3) Sputum
- (4) Blood by venipuncture

b. Investigations initiated:

- (1) Urine - microscopy\*
  - quantitative bacterial culture $\phi$
- (2) Throat swab - bacterial culture $\phi$
- (3) Sputum - bacterial culture $\phi$
- (4) Blood - I/P mouse inoculation\*
  - bacterial culture $\phi$
  - full blood count\*
  - serum separation and freezing\*
  - 1-2 mls fresh whole blood preserved in liquid N<sub>2</sub>
  - Slide serology: Widal 'O'
    - Weil-Felix OXK\*
    - C-reactive protein\*
    - latex agglutination
  - Thick film for MP's\*

\* Completed locally by unit satellite laboratory.

$\phi$  Initial readings completed by local unit satellite laboratory.

c. Base laboratory investigations:

- (1) Confirmation of bacterial culture readings
- (2) Typing and sensitivities of bacterial cultures

- (3) Febrile agglutinin serology - Weil-Felix OXK, OX2 and OX19; Widal 'O' and 'H'; Leptospirosis HL; melioidosis HA; brucellosis HA.
- (4) IFAT for 9 strains of scrub typhus, STT and endemic typhus.
- (5) Isolation procedures on inoculated mice.

d. Treatment given as directed by, and at the discretion of the doctor in clinical charge. An advisory schedule using diagnostic probabilities is supplied to him as an aid.

3. Continuation Procedures:

a. Day 2: The local unit laboratory will make initial readings of bacterial cultures and make indicated subcultures for identification and/or sensitivity testing as necessary.

b. Day 3: Patient seen routinely and any indicated adjustment to treatment made. Blood sample taken by venipuncture for serum and, if patient febrile, for second blood culture. Routine thick film for MP's and sequestrine sample for full blood count will be taken in all cases. Slide serology to be performed as earlier and frozen serum sent to the base laboratory for performance of febrile agglutinin and IFA serology.

c. Days 7 and 14: Follow-up samples of blood for base laboratory serology (febrile agglutinins and IFAT), plus (on day 7 only) latex agglutination CRP slide test to be performed locally.

4. Documentation: All data is recorded in ADP compatible format. Data includes initial clinical data and serial presumptive diagnostic and therapeutic data as well as the complete results of laboratory investigations.

5. Patient Disposal: The timing and route of patient disposal is recorded. At all stages of the febrile episode and its follow-up there is the option of hospital admission by the decision of the Medical Officer in clinical charge; the study site is in the catchment area of the district hospital at Mentakab where unit facilities exist and there is full cooperation from the Consultant Physician in charge - such patients remain, therefore, an integral part of the study and without loss of data.

Study Coordination: This study is part of an interlocking series of studies being conducted in parallel. It is emphasized that this project will be the main source of isolate material and sera and will supply such specimens at an estimated rate four times that of the hospital based PUO study. Most importantly it is supplying human origin specimens from the same areas where distribution of

R. tsutsugamushi in vectors and reservoir hosts is being studied along with factors influencing transmission of the organism. It is the study by which much of the data obtained through various projects are integrated and together with the longitudinal study in the same area will enable interpretation of the mass of generated information in respect of human disease and infection.

6. Current Status: After a slow start due mainly to seasonal influences, the study is well under way and will obviously be a source of much valuable information. It is already apparent that there is a greater than expected incidence of typhoid and leptospirosis plus the medical problems associated with an unsafe water supply. A side-issue (but of considerable PR importance) is the identification of epidemic scabies and a widespread skin sensitization problem that is probably associated with certain ground cover plants.

#### A LONGITUDINAL STUDY OF THE CHARACTERISTICS OF R. TSUTSUGAMUSHI INFECTION AMONG FELDA OIL-PALM SETTLERS IN WEST PAHANG.

This study was initiated in April 1977 using a carefully planned and detailed schedule.

There are two areas where information is required: firstly the comparison of disease, infection and recrudescence (or reinfection) rates to produce a realistic assessment of risk and secondly to relate infection and disease rates to influencing factors (e.g. ecological, meteorological, chigger species, chigger infectivity, organism strains etc.) to give some "index of risk". There is also the necessity to firmly identify not only a generic "high risk population" but also the prediction of infection/disease pattern within that population when the time comes for human trials of prophylactic agents of any nature.

The studies at Bukit Mendi together with the limited extrapolation that can be made from some of the hospital-based PUO study material, would seem to indicate that the combination of a susceptible population and its location in a high risk area only prevails for a limited time (approx. 1-2 yrs) in the first 3 years of a FELDA oil palm settler's life. A new scheme was identified in the Jengka triangle suitable for longitudinal study.

The servicing of the study is amalgamated with the rural health center PUO study at Jengka. The study itself is in three phases.

1. A complete cross-sectional survey of settlers on two schemes within Jengka. One (Jengka 15) is an oil-palm settlement of some 340 families (total population with dependents approx. 2000)

on which settlement began in October 1976 and is now largely complete. It is expected that this population is susceptible and that the vector chiggers have not yet become fully established within the scheme. The initial serological, isolation, hematological and social data provide a base line against which to judge events and changes. The other (Jengka 11) is an oil-palm settlement of some 450 families (total population over 3000) which is about 15 months older than Jengka 15. Approximately 350 settlers are 'original' and it is known that clinical cases of scrub typhus have occurred among them in recent months - and the remainder settled during the same period as the Jengka 15 population. In this settlement, therefore, there is a risk and part of the population is (probably) highly susceptible and part less so.

2. The Jengka 15 population has monthly sample surveys of approximately 15% of the population; selection is by random number (using the settler identification number as the index). Selection will be exclusive. Information collected at each sample survey includes serological, hematological, isolation (?) and social/work data.

3. The Jengka 15 settlers are monitored at the daily roll call for sickness absence in an effort to detect all febrile illnesses and obtain a causal diagnosis. By this means and the random surveys the pattern of infection and disease with respect to scrub typhus will emerge.

Contemporaneously with these exercises, information concerning influencing factors is being gathered in other studies thus enabling the production of the risk index. Additionally, this is the first attempt to study and distinguish between infection and disease incidence in a population that eliminates (as far as is ever possible) uncontrolled effects of selection and competing health care delivery systems.

Current Status: In Jengka 15 as of 30 September 1977, the fourth sub-sample survey was well underway, the initial cross-sectional and first three sub-sample surveys having achieved a full-response rate well excess of 90% on each occasion. The serology shows (as was hoped) a low level of previous exposure to R. tsutsugamushi and no cases have occurred on site up to 30 September 1977.

The initial survey on Jengka 11 is virtually complete but results are not yet available.

## IDENTIFICATION OF RICKETTSIA TSUTSUGAMUSHI IN NATURALLY INFECTED CHIGGERS (ACARINA: TROMBICULIDAE) BY DIRECT IMMUNOFLUORESCENCE.

The detection of rickettsial organisms in different vectors has depended primarily on the clinical and serological findings in susceptible laboratory animals. However, animal isolation procedures are long and laborious, often requiring 2-3 months for final confirmation.

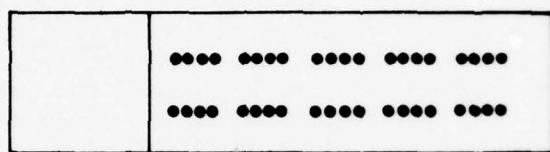
Coons and Kaplan (21) first reported the use of a fluorescent antibody (FA) technique to detect rickettsial organisms in an arthropod vector when they identified epidemic typhus organisms in a smear prepared from a human body louse, *Pediculus humanus humanus*. The detection of Rocky Mountain spotted fever organisms in the wood tick, *Dermacentor andersoni*, by means of a direct FA method was subsequently reported by Burgdorfer and Lackman (16) and by Shepard and Goldwasser (88). Mirolyubova et al. (72) described the use of an indirect FA test to determine the natural infection of *Rickettsia tsutsugamushi* in trombiculid mites. Iida et al. (53) first demonstrated the use of the direct immunofluorescent technique as a method of typing strains of *R. tsutsugamushi*. Recent reports from this unit have demonstrated the use of the direct FA technique to study the distribution of scrub typhus organisms in organs of *Leptotrombidium* (*Leptotrombidium*) *fletcheri* mites (78, 80). This report describes the application of a direct FA test to identify scrub typhus infection and the strain(s) of the infecting organisms in a chigger vector.

### Mites

Infected mites from previously described laboratory colonies of *L. (L.) fletcheri* (76) and of *L. (L.) arenicola* (79) were used throughout this study. Different developmental stages of mites were examined: unengorged and engorged larvae, nymphophanes, nymphs, telieophanes and adults. Additionally, uninfected mites of both species from laboratory maintained colonies were also used.

### Preparation of Slides

Each mite was placed in approximately 5 lambda of 0.5% normal yolk sac suspension, which was a drop dispensed from a tuberculin syringe with a 26 gauge needle. Under a dissecting microscope, the exoskeleton of the mite was punctured dorsal-posteriorly, and the internal contents squeezed out using 2 minutin pins. The contents were then mixed with the normal yolk sac suspension. Spots of the mite suspension were applied to the predetermined areas on a pre-cleaned microscope slide by using a pen nib (Kingsley 2788; Hinks, Wells & Co., England). Suspensions for 4 specimens were placed on each slide (Figure 1). The spots were



•  
1    2    3    4  
SPECIMENS

Figure 1

dried at room temperature for a minimum of 30 minutes before fixation with carbon tetrachloride for 10 minutes. The fixed slides were again dried at room temperature. The slides were normally read immediately, but they could be stored at -20°C for as much as 1 month without noticeable decrease in intensity of fluorescence.

The exoskeleton of the chigger remained sufficiently intact and was mounted in a drop of Hoyer's mounting media (Ward's, Rochester, New York) for species identification.

#### Conjugates

The strain-specific fluorescein conjugates were prepared in rabbits by the Department of Rickettsial Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20012. The 9 strains of *R. tsutsugamushi* used in the preparation of conjugates in this study have been previously described. The Karp, Gilliam and Kato strains were established as the 3 prototype scrub typhus strains by utilizing the purified CF antigens (89, 90). Antigenic analysis of many strains isolated from different sources in Thailand has resulted in the recognition of 5 other distinctive strains: TA 678, TA 686, TA 716, TA 763 and TH 1817 (32). The strain, TC 586, antigenically indistinguishable from the Gilliam strain, was evaluated as a substitute for the Gilliam strain in experimental vaccines (29).

#### Technique of Direct Immunofluorescence

If the fixed slides had been stored at -20°C, they were warmed to room temperature before use to remove any condensate. Groups of 4 spots on the slides were ringed with nail polish, and 0.005 to 0.01 ml of the optimal working dilutions of the strain-specific conjugates were applied to the respective areas (Figure 2). The slides were then incubated 35-37°C in a moist chamber for 30 minutes, washed 2 times (3 minutes each) with phosphate buffered saline (PBS), pH 7.3, and air-dried. To all spots were then added 0.005 to 0.01 ml of the bovine albumin rhodamine labeled counterstain (BBL, Cockeysville, Maryland 21030), which was diluted 1:20 with PBS. The slides were incubated at 35-37°C for 30 minutes, after which they were washed 2 times (3 minutes each) with PBS and air-dried. The slides were mounted under cover slips in buffered glycerine, pH 7.3. Stained preparations were examined at 125X and 500X magnifications under a Leitz Orthoplan microscope converted to vertical illumination with a HBO mercury vapor lamp, using BG-12 and S-400 exciter filters. The beam splitting device built into the vertical illuminator gave an effective dichroic combination of a 455 non-reflectance and a K-460 suppression filter. Additional K-590 and K-610 suppression filters were added to the system. The slides were usually read within 24 hours, although the results were not

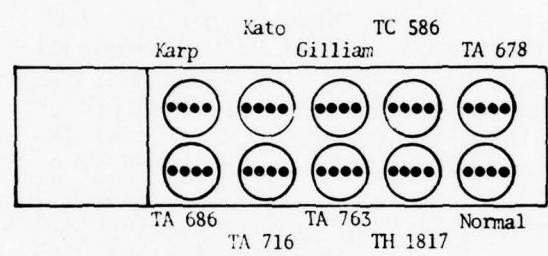


Figure 2

altered when stored for 2 days at 4°C. A positive reaction was graded according to the intensity of specific fluorescence and the number of organisms with typical rickettsial morphology in a specimen.

The *R. tsutsugamushi* organisms that reacted specifically with the fluorescein-conjugated antibody stained a bright yellow-green, whereas the background material usually appeared a dull yellow in color.

Rickettsial organisms were observed in all stages, but with greatest frequency and intensity in the unengorged larvae. Additionally autofluorescing bodies were minimal in this stage. Because of this unengorged larvae of both *L. (L.) fletcheri* and *L. (L.) arenicola* were used in evaluation of this technique.

Three hundred sixty of the infected *L. (L.) fletcheri* chiggers and 400 of the infected *L. (L.) arenicola* chiggers were tested and both species were found to carry scrub typhus rickettsiae antigenically related to Karp, TA 686, TA 716, and TA 763. Additionally some *L. (L.) fletcheri* chiggers were found to contain rickettsia which reacted with Gilliam strain antisera. Studies are underway to define the antigenic characteristics of the strains caused by these 2 species of chiggers. Smears from more than 200 uninfected chiggers of both species did not react with any of the 9 conjugates.

A total of 100 coded infected and uninfected larval specimens from both species were diagnosed. Of 60 infected chiggers, 56 were correctly identified. The uninfected chiggers were identified in all cases. Some infected larvae were probably not identified due to the small numbers of rickettsial organisms present in the preparation. In most cases, the intensity of the specific fluorescence and the number of the rickettsiae were quite satisfactory.

The direct immunofluorescent technique can be used to detect scrub typhus rickettsiae in naturally infected mites. Traub and Wisseman (93) pointed out that non-specific autofluorescence of mite tissues occurred in previous studies using the FA test. During the examination of different stages of the mite in our study, only the unengorged larvae and telioophanes demonstrated minimal autofluorescence, allowing for easy detection of the organism.

It is fortunate that the infection can be noted easily in the unengorged larval stage, as this is perhaps the most important stage to consider when conducting vector surveys or infectivity studies. Trombiculid mites feed on mammals only during the larval stage and, unlike many other parasitic arthropods, they usually feed only once during this stage. Thus,

in order for a chigger to act as a vector, it must be infected prior to the larval stage. Walker et al. (98) demonstrated that chiggers can acquire the infection from infected rodents and transmit it transtadially through the adult stage. Therefore, examination of engorged larvae and post-larval stages may simply reflect the infection within the host and not the potential of the chigger to act as a vector. Many of the previous reports of chiggers infectivity have been from engorged chiggers collected from their hosts.

Presently, unengorged chiggers collected from various study sites are being examined for their infectiveness. The rapidity in obtaining the results makes the immunofluorescence method much superior to the time-consuming isolation procedure in mice. With the mouse isolation technique the results are often delayed by as much as 3 months, while with this technique we can collect the chiggers, test them for infectivity, and identify them to species within 2-3 days.

The accuracy of this direct immunofluorescence technique compares favorably with the 2 previous studies using the mouse isolation technique for identification of infectivity within chiggers. In these 2 studies, utilizing L. (L.) fletcheri chiggers from the same colony as used in this study, not all individuals that were fed transmitted infection. Rapmund et al. (76) reported a 91.6 (515 of 569) percent infectivity rate in 4 generations (P-F<sub>3</sub>), while Roberts et al. (78) reported a 95.0 (19 of 20) percent infectivity rate of a later unspecified generation. Rapmund et al. (76) postulated that "the apparent failure of these individuals to transmit rickettsiae during feeding as larvae may be genuine, even though as adults they passed rickettsiae transovarially, and may exemplify an event occurring in nature. Alternatively, rickettsiae may have been transmitted during feeding but our rickettsial isolation techniques failed to detect them." We feel that, this study indicates that the technique is sensitive enough to utilize for field surveys.

The adaptability of the direct immunofluorescence technique for the identification of infection within chiggers will facilitate epidemiological studies of scrub typhus and will allow numerous basic questions regarding the infectivity of natural populations to be answered. Specific questions that can be undertaken include: What is the natural infection rates within vector chiggers? Are infection rates different in different habitats? What are the predominant strains of R. tsutsugamushi in nature? Is there geographical variation between strains and infectivity? With answers to these and other questions that can be answered with the direct immunofluorescence test, a means of predicting highly endemic areas or habitats may be developed.

## EPIDEMIOLOGY OF SCRUB TYPHUS ON A MATURE OIL-PALM ESTATE

The assessment of risk to humans of contracting Rickettsia tsutsugamushi infection in defined habitats has not been adequately examined. Furthermore, no correlation has been established between infection in chiggers and that of mammalian hosts. The importance of small rodents as maintaining hosts for chiggers has been proven on many occasions. The chief purpose in studying rodent hosts is to assess the potential of infection for humans through extrapolation of the rodent data. Although a definitive relationship has not been established between rodent and human infections, rodents have been widely used to study the epidemiology of R. tsutsugamushi. Elmina Estate afforded an opportunity to compare the incidences of infections in chiggers with those in rodents within a circumscribed area.

Elmina Estate is located approximately 28 miles NW of Kuala Lumpur, Peninsular Malaysia. The estate consists of 2181 acres of oil-palm and 1239 acres of rubber. The study was conducted in an area containing fully mature oil-palm trees of about 25-30 years old. The floor of the study area was covered with short weeds and creepers. An area of about 2 meters in diameter around each tree was completely cleared and maintained by the estate workers who regularly prune the tree. The fronds were piled in rows one meter away from the tree. The field rat, Rattus tiomanicus, build their nests under the heaps of the fronds and also on the trees.

A grid of 200 points spaced out at 25 feet intervals from the east-west and north-south axis was mapped within the study area (Figure 3). Two hundred traps were used and each trap was placed at each point of the grid. Trapping of animals in the grid was carried out on the first week of each month from December 1974 through December 1976, with the exception of July through October 1976. All animals trapped were brought back to the laboratory for examination. The animals were anesthetized and the ectoparasites removed. Two ml of blood were taken directly from the heart. The animals were then marked by clipping the toes and released the next day at the site of capture. The catch on the last day of the week was sacrificed, and the tissues were processed for isolation.

Whole blood or tissue specimens from the rodents were inoculated intraperitoneally into pairs of laboratory mice. Subsequent passages were made, and the isolation of R. tsutsugamushi was confirmed by challenging the mice with the virulent Karp strain of R. tsutsugamushi (11). Antigenic characterization of the isolates was performed by direct immunofluorescence.

Two transects of 11 points each were established through the grid (Figure 3). Initially specific areas were searched until

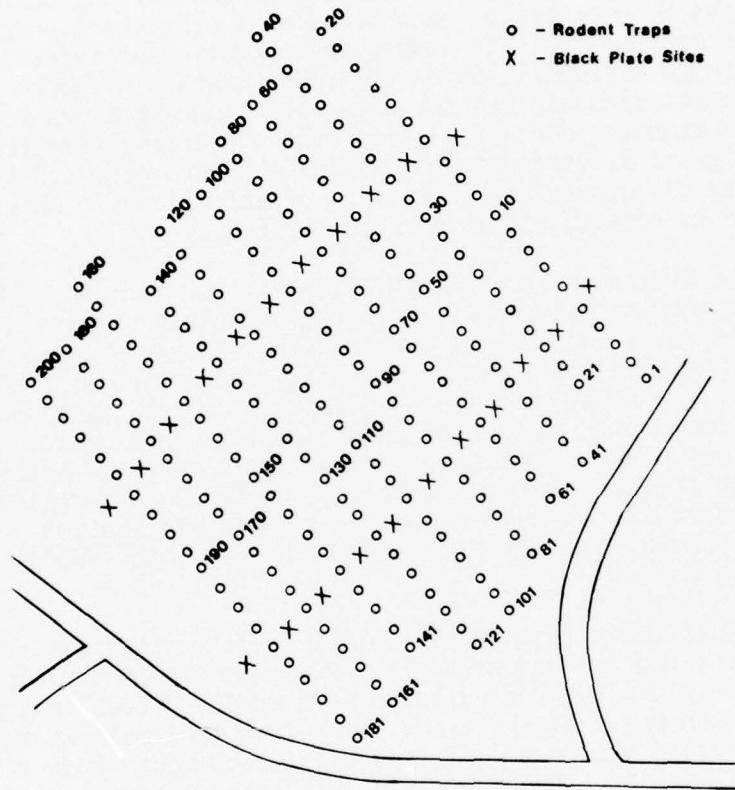


Diagram of Trapping Grid and Black Plate Sites in  
 a Mature Oil Palm at Elmina Estate, Selangor,  
 Peninsular Malaysia.

Figure 3

chiggers were found and the site established. A stake was placed at this point and monthly collections were made at this exact site. These sites were left as undisturbed as possible. Each month 10 black plates (39) were used for chigger collection at each site. The chiggers were collected and returned to the laboratory for identification.

In the early part of the study, rickettsial isolation from chiggers was performed as described by Jackson et al. (55). Rickettsia tsutsugamushi infections in chiggers collected after mid-1976 were determined by the direct fluorescent antibody technique which was simultaneously used to antigenically characterize the strains.

During the period from December 1974 through December 1975, a total of 644 field rats, R. tiomanicus and 11 tree shrews, Tupaia glis, were trapped (Table 1). The isolation rates were 0.3% (2/644) for R. tiomanicus and 9.1% (1/11) for T. glis. None of the 2 common squirrels (Callosciurus notatus), one house rat (R. rattus diardii), and one ricefield rat (R. argentiventer) trapped in the same area were found to be infected with R. tsutsugamushi.

In 1976, 293 R. tiomanicus and 4 T. glis were collected (Table 2). No trapping was done from July through October. Only the field rats were found to be infected, with 1.7% (5/293) isolation rate. Attempts to isolate rickettsiae from T. glis were unsuccessful.

Thus far, 4 of the 8 isolates have been characterized serologically, and the following results were obtained: one was antigenically related to the Karp strain; one reacted to both Karp and TA 763 strains; one to TA 686, TA 716 and TA 763 strains; and one to the strains of Karp and Gilliam.

Chiggers were collected from R. tiomanicus from December 1974 through May 1975. Scrub typhus organisms were isolated from only one of 151 (0.7%) pools of chiggers. The single positive pool was collected in December 1974.

From July 1976 through February 1977, 3 species of chiggers, L. (L.) deliense, L. (L.) fletcheri, and L. (L.) vivericola, were collected by the black plate technique. R. tsutsugamushi organisms were identified in 1.8% (10/570) L. (L.) deliense and 1.3% (1/80) L. (L.) vivericola (Table 3). No organisms were observed in any of the L. (L.) fletcheri mites.

Antigenic characterization of the organisms in the chiggers collected in 1976/1977 showed 10 related antigenically to the Karp strain and one to TA 716 strain. The one identified in L. (L.) vivericola was related to the Karp strain. The single isolate from 1974 is presently being characterized.

Table 1. Isolation of scrub typhus organisms in Rattus tiomanicus and Tupaia glis trapped at Elmina Oil-Palm Estate in 1974/1975.

Month	<u>Rattus tiomanicus</u>		<u>Tupaia glis</u>	
	Pos/Total*	% Pos	Pos/Total	% Pos
Dec 74	0/41	0	0/0	0
Jan 75	0/66	0	1/3	33.0
Feb	0/31	0	0/0	0
Mar	0/37	0	0/1	0
Apr	0/48	0	0/0	0
May	0/81	0	0/0	0
Jun	0/112	0	0/2	0
Jul	0/65	0	0/0	0
Aug	0/48	0	0/1	0
Sep	0/44	0	0/1	0
Oct	0/20	0	0/0	0
Nov	1/29	3.4	0/0	0
Dec	1/22	4.5	0/3	0
Total	2/644	0.3	1/11	9.1

\* Pos/Total = Number of animals from which scrub typhus organisms were isolated/total number of animals.

Table 2. Isolation of scrub typhus organisms in Rattus tiomanicus and Tupaia glis trapped at Elmina Oil-Palm Estate in 1976.

Month	<u>Rattus tiomanicus</u>		<u>Tupaia glis</u>	
	Pos/Total*	% Pos	Pos/Total	% Pos
Jan 76	0/15	0	0/1	0
Feb	1/19	5.3	0/0	0
Mar	0/24	0	0/0	0
Apr	1/33	3.0	0/1	0
May	1/28	3.6	0/0	0
Jun	1/69	1.4	0/2	0
Jul	-**	-	-	-
Aug	-	-	-	-
Sep	-	-	-	-
Oct	-	-	-	-
Nov	1/63	1.6	0/0	0
Dec	0/42	0	0/0	0
Total	5/293	1.7	0/4	0

\* Pos/Total = Number of animals from which scrub typhus organisms were isolated/total number of animals.

\*\* No animals processed for isolation.

Table 3. Identification of scrub typhus organisms in different species of vector chiggers collected at Elmina Oil-Palm Estate in 1976/1977.

Month	<u>L.</u> ( <u>L.</u> ) <u>deliense</u>		<u>L.</u> ( <u>L.</u> ) <u>fletcheri</u>		<u>L.</u> ( <u>L.</u> ) <u>vivericola</u>	
	Pos/Total*	% Pos	Pos/Total	% Pos	Pos/Total	% Pos
Jul 76	0/25	0	0/0	0	0/0	0
Aug	3/65	4.6	0/14	0	0/2	0
Sep	0/26	0	0/0	0	0/2	0
Oct	0/0	0	0/0	0	0/0	0
Nov	0/165	0	0/0	0	0/43	0
Dec	4/218	1.8	0/0	0	0/29	0
Jan 77	2/54	3.7	0/0	0	1/4	25.0
Feb	1/17	5.9	0/0	0	0/0	0
Total	10/570	1.8	0/14	0	1/80	1.3

\* Pos/Total = Number of chiggers in which scrub typhus organisms were identified/total number of chiggers.

Throughout the 22 months of this study, 100,702 chiggers were collected from rodents. Of these 21.5 percent (21,639) were mounted for identification. Eleven species of chiggers were identified, including: L. (L.) deliense (63.7%), L. (L.) fletcheri (0.12%), L. (L.) vivericola (0.02%), L. (L.) bodense (0.12%), Ascacioengastia indica (31.05%), A. loriensis (0.05%), Walchia lewthwaitei (3.88%), W. enode (0.01%), W. ewingi (0.02%), Gahrliepia fletcheri (0.42%) and Walchiella impar (0.05%).

Figure 4 presents a comparison of the number of L. (L.) deliense per R. tiomanicus (excluding rats recaptured during the same collection period) and the number of L. (L.) deliense per black plate. In general, the two lines tend to follow the same pattern. The number of chiggers per black plate collected during February 1975, may be considered artificially high as a site was not established until at least one chigger was collected from the site.

#### EPIDEMIOLOGY OF SCRUB TYPHUS ON A MALAYSIAN OIL-PALM DEVELOPMENT

Scrub typhus has been known to be prevalent amongst Malaysian oil-palm workers for 50 years (36). More recently introduced techniques, such as isolation of Rickettsia tsutsugamushi by mouse inoculation (31), and direct (53) and indirect immunofluorescence (8), have enabled this laboratory to identify scrub typhus as a leading cause of illness in rural Malaysia, and particularly in oil-palm workers. In contrast to the 46 cases of scrub typhus reported (personal communication, Dr. J.S. Gill, Assistant Director of Health Services) to the Ministry of Health, Malaysia in 1974, we identified 58 cases, occurring in 6 months, on one oil-palm scheme alone (11).

Since large areas of Malaysian rainforest are being cleared for oil-palm production, and hundreds of thousands of people settled on these plantations, the potential number of infections is very large. The infection is known (36) to be distributed patchily in oil-palm, depending on localised ecological conditions. It therefore seemed important to identify, using the new technology, conditions giving rise to a high prevalence of infection.

The existence of multiple serotypes of R. tsutsugamushi, within a small area, has been reported from Malaya (71) and 5 strains distinct from the prototype Karp, Kato and Gilliam were found in Thailand (33). Characterization of isolates causing human disease is essential for an understanding of any immunity

Number of Leptotrombiculum (L) deliense per Trapped Rattus  
tiomanicus Compared with the Number of L (L) deliense  
per Black Plate - Elimina Estate.

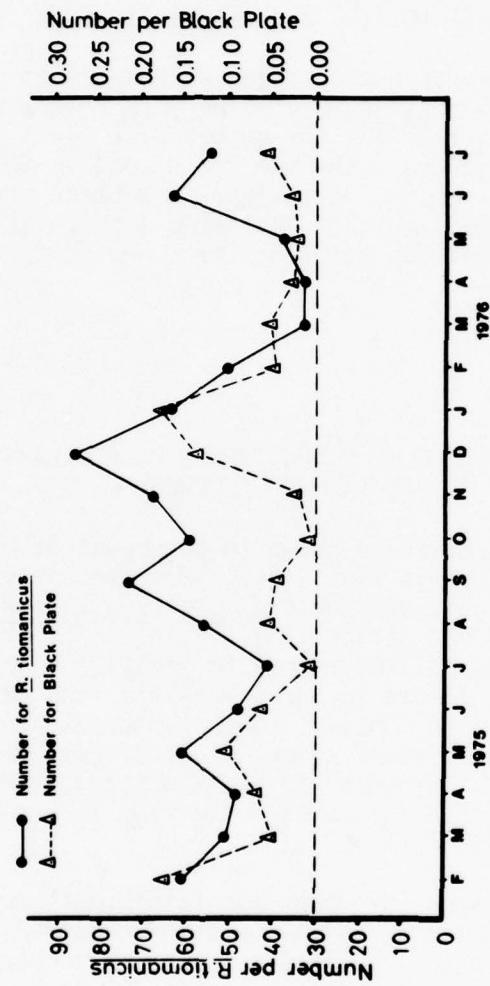


Figure 4

to re-infection, and is a pre-requisite to any attempt at vaccine production.

We report here a study of the epidemiology of scrub typhus on the oil-palm scheme at Bukit Mendi.

The Federal Land Development Authority (FELDA) oil-palm complex at Bukit Mendi is located in the middle of a triangle formed by Karak, Temerloh and Kuala Pilah, just south of the Kemasul Forest Reserve in southern Pahang (Figure 5). It consists of 4 schemes, one of which also bears the name Bukit Mendi, and the data presented here relates to that scheme only. All the workers on the scheme live in Bukit Mendi village, with a total population of approximately 2500 at the time of the study. The land was cleared, planted and settled in 3 phases, each one year apart. Phase I of Bukit Mendi at the beginning of the study was approximately 7 years old, while Phase III was only approximately 5 years old. The village also contained a processing factory, offices, shop-houses and schools, so not all residents were equally exposed to infection in the plantation.

The establishment of a FELDA oil-palm scheme includes first the clearing of the existing forest and then the planting of the oil-palm by contract laborers. Settlers are not brought into the scheme until the oil-palm trees are approaching bearing age, which is approximately 4-5 years after planting. The settlers are provided with a house and approximately 10 acres of oil-palm trees. Each family is responsible for the complete maintenance of his acreage, including weeding, fertilizing, pruning, and collection of the fruit. A division of labor, as is often seen in privately owned estates, is not established.

### 1. Human Infections

From March 1975 to August 1976, febrile patients attending the Bukit Mendi rural health centre was studied. Demographic, epidemiological and limited clinical data was collected, and acute and convalescent specimens of blood collected.

Sera were stored at -20°C from the time of separation, which was carried out at the health center. Paired samples were examined simultaneously for antibody to the known 9 strains (33) of *R. tsutsugamushi*, by indirect fluorescent antibody technique (IFA)(81). The strains (Karp, Kato, Gilliam, TC 586, TA 678, TA 686, TA 716, TA 763 and TH 1817) were obtained from the Department of Rickettsial Diseases, Walter Reed Army Institute of Research.

Freshly drawn venous blood specimens were inoculated intraperitoneally into pairs of mice, at the study site. Material from these mice was subsequently passaged into further

**Location Of Bukit Mendi, FELDA Oil Palm Complex,  
Pahang, Peninsular Malaysia.**



1 in = 12 miles

Figure 5

mice, and any isolates identified by challenge with a virulent strain of R. tsutsugamushi (11).

An otherwise unexplained illness plus one or more of the following criteria was considered evidence of infection:

- (i) isolation of the organism
- (ii) a 4-fold or greater rise in IFA titer to at least one antigen.

A static IFA titer of 1/400 or more was regarded as suggestive of recent or concurrent infection.

The incidence of infection is tabulated, according to the recorded place of exposure, in Table 4. Overall, 23 (11.5%) of the 200 people who admitted working in the plantation were definitely infected, and probably a further 9 (4.5%), who had high titers but no rise. Two of the 4 definite cases among the village group had been into the field, but their exact place of exposure was unknown.

There was a significantly greater incidence of scrub typhus cases among the Phase III workers than those in Phases I and II, and a significantly smaller incidence in the village group (excluding the 2 exposed in the field).

A cluster of cases occurred in Phase III during the period June-August 1975, when isolates were obtained from 8 patients, 7 of whom were female. Three of these had been working in one 200 acre block, 3 in an adjacent 150 acre block, and the remaining two in other areas.

A breakdown of cases by age groups and sex is shown on Table 5, which includes only those definite cases occurring among plantation workers.

## 2. Rodent and Chigger Infections

Scrub typhus has been shown to be a common cause of febrile illness in rural Malaysia and has been especially prevalent among oil-palm workers (11). Since rodents abound in these areas and act as hosts for the vector chiggers, it was decided to study the relationship between strains existing in nature and those causing human infections. In other words, can relationships be established between infections in chiggers, in rodents, and in humans? Of the many different study sites, Bukit Mendi was the most ideal site to carry out an integrated study to answer the question. This report presents the data on the isolates from rodents and vector chiggers collected at this particular site.

Table 4. Incidence of scrub typhus in relation to exposure

	Phase			Village	Total
	I	II	III		
Isolate	1	2	11 <sup>1</sup>	3 <sup>2</sup>	17
Seroconversion	3 <sup>3</sup>	4 <sup>3</sup>	2	1 <sup>2,3</sup>	10
Static titer >1/400	6	1	2	2	11
Total subjects presenting	67	85	48	113	313
Family units at risk	137	221	71	- <sup>4</sup>	-

1. 8 isolates obtained during June-August 1975, 7 of them from females.
2. 1 patient in each group had been exposed in numerous parts of the plantation.
3. Maximum IFA titer 1/100 in 1 patient in each group.
4. Data not available.

Table 5. Incidence<sup>1</sup> of scrub typhus by age and sex

	Age Group				Total
	<10	10-14	15-44	>44	
Male	0/17	1/9	9/91	0/3	10/120
Female	0/7	1/8	12/54	0/2	13/71
Total	0/24	2/17	21/145	0/5	23/191

1. Field workers only. Isolate and or seroconversion/total in each group.

The Bukit Mendi complex in southern Pahang has been previously described by Brown et al. (11). Phases I and III, the sites of this study, have been described above. For rodent study, 2 trapping grids of 100 traps each was established in Phase I (Blocks 7 and 9), and both were located in oil-palm habitats. In Phase III, 2 trapping grids of 100 traps each were also established; however, one (FF) was established on the forest fringe, so that 50 traps were in the forest habitat and the other 50 in the oil-palm habitat. The other grid (YY) was exclusively in the oil-palm area. All grids were trapped for one week each month. All rodents trapped in Phase III and block 7 of Phase I were bled via cardiac puncture in the field and released at the site of capture. The animals trapped from the other block in Phase I were returned to the laboratory for identification and bleeding.

For collecting chiggers by black plating, sites were established within the grids of both Phase I and Phase III. Ten plates per site were examined monthly and randomly selected chiggers were returned to the laboratory for determining the presence of Rickettsia tsutsugamushi organisms.

Strain characterization of the organisms in the chiggers was performed by the direct fluorescent antibody technique. The strain-specific fluorescein conjugates used in direct immunofluorescent tests were prepared using sera collected from rabbits infected with Karp, Gilliam, Kato, TC 586, TA 678, TA 686, TA 716, TA 763, and TH 1817.

The blood or tissue specimens from the rodents were injected intraperitoneally into pairs of laboratory mice, and further processing to determine the presence of R. tsutsugamushi in the original inoculum was done by previously described procedure (11). Characterization of the isolates was performed by the direct immunofluorescence technique.

For isolation study, systematic trapping of animals in Phase III was begun in January 1976, while that in Phase I was started in September 1976. During this period, the most common animal was Rattus tiomanicus, comprising 75% (234/312) of the total animals trapped in the study area. It was noted that no apparent seasonal infections occurred among the animals within the grids (Table 6). Rickettsia tsutsugamushi organisms were isolated from 5 different animals: R. tiomanicus - 20.9% (49/234); R. argentiventer - 26.1% (6/23); R. exulans - 25.0% (1/4); Callosciurus notatus - 14.3% (2/14); and Tupaia glis - 11.1% (1/9) (Table 7). Attempts to isolate organisms from 7 other rodent species were unsuccessful. A greater number of different species of animals were collected in the FF grid, because of the diverse habitats existing within the area.

Table 6. Isolation of scrub typhus organisms in animals trapped at Bukit Mendi oil-palm scheme.

Date	PHASE I (7)		PHASE III (FF)		PHASE III (YY)	
	Pos/Total*	% Pos	Pos/Total	% Pos	Pos/Total	% Pos
Jan 76	-	-	2/14	14.3	4/18	22.2
Feb	-	-	5/18	27.8	1/5	20.0
Mar	-	-	2/24	8.3	3/6	50.0
Apr	-	-	1/16	6.3	1/4	25.0
May	-	-	6/21	28.6	0/5	0
Jun	-	-	2/7	28.6	1/7	14.3
Jul	-	-	0/8	0	0/5	0
Aug	-	-	3/7	42.9	1/3	33.3
Sep	0/4	0	7/19	36.8	1/5	20.0
Oct	0/10	0	3/10	30.0	2/6	33.3
Nov	1/10	10.0	0/8	0	2/6	33.3
Dec	1/8	12.5	3/7	28.6	1/5	20.0
Jan 77	0/3	0	2/10	20.0	1/9	11.1
Feb	0/8	0	1/7	14.3	3/9	33.3
Total	2/43	4.7	36/176	20.5	21/93	22.6

\* Pos/Total = Number of animals from which scrub typhus organisms were isolated/total number of animals.

Table 7. Isolation of scrub typhus organisms from different animals trapped at Bukit Mendi oil-palm scheme

Animals	PHASE I (7)		PHASE III (FF)		PHASE III (YY)	
	Pos/Total*	% Pos	Pos/Total	% Pos	Pos/Total	% Pos
<u>Rattus tiomanicus</u>	2/41	4.9	32/118	27.1	15/75	20.0
<u>Rattus argentiventer</u>	-	-	1/9	11.1	5/14	35.7
<u>Rattus whiteheadi</u>	-	-	0/5	0	-	-
<u>Rattus muelleri</u>	-	-	0/4	0	-	-
<u>Rattus exulans</u>	-	-	1/1	100.0	0/3	0
<u>Rattus sabanus</u>	-	-	0/1	0	-	-
<u>Echinosorex gymnurus</u>	-	-	0/4	0	-	-
<u>Callosciurus notatus</u>	-	-	2/14	14.3	-	-
<u>Sundasciurus lowii</u>	-	-	0/1	0	-	-
<u>Chiropodomys gliroides</u>	-	-	0/1	0	-	-
<u>Sundasciurus tenuis</u>	-	-	0/12	0	-	-
<u>Tupaia glis</u>	0/2	0	0/6	0	1/1	100.0
Total	2/43	4.7	36/176	20.5	21/93	22.6

\* Pos/Total = number of animals from which scrub typhus organisms were isolated/total number of animals.

Thirty seven of the 59 isolates have currently been characterized serologically (Table 8). Antigens related to the Karp strain were found in 94.6% (35/37) of the isolates, while 10.8% (4/37) carried Gilliam-related antigen. However, 2 had only Gilliam and TC 586 antigens, the latter being serologically indistinguishable from the Gilliam strain. The different antigenic strains appear to exist rather uniformly throughout the 2 different grids of Phase III (Table 9). The breakdown of the strains among the different groups of animals from which the isolates were made showed no specific pattern (Table 10).

Only 2 species of mites, Leptotrombidium (L.) deliense and L. (L.) vivericola, were collected by the black plate technique throughout the study. It is interesting to note that 98.5% (1678/1703) of the L. (L.) deliense chiggers were collected from Phase III, while all but one L. (L.) vivericola were from Phase I. Scrub typhus organisms were identified in 80 of 1703 (4.7%) L. (L.) deliense chiggers, of which only one was from Phase I (Table 11). Of the remaining 79 positives chiggers found in Phase III, 21.5% (17/79) were collected in YY grid and 78.5% (62/79) in FF grid. Fifteen of 358 (4.2%) L. (L.) vivericola chiggers were found to be infected with R. tsutsugamushi organisms.

Strain characterization of these isolates demonstrated that all reacted antigenically to the Karp strain, either singly or multiply with other Karp-like strains (Table 12).

### 3. Ecological Considerations

As was previously reported (Walter Reed Army Institute of Research Annual Progress Report 1 July 1975 - 30 June 1976), an initial survey of the rodents and their chiggers from different habitats throughout the entire complex was conducted. The predominant rodent species throughout all habitats was R. tiomanicus. L. (L.) deliense was the predominant species of chiggers from 4 specific types of habitat: lalang (76.8%), forest fringe (49.8%), Phase I oil-palm (50.6%) and Phase III oil-palm (72.6%).

As reported above, a larger number of cases of scrub typhus occurred within the younger oil palm (Phase III) than within the older oil-palm (Phase I). In an effort to determine what reason might be causing such a pattern of infection rates, study sites within both Phase I and III were established. Two trapping grids were established in each Phase. Of the 4 sites studied, 2 in Phase I (Block 7 and 9) and 1 in Phase (YY) consisted entirely of oil-palm, while the remaining site in Phase III (FF) was made up of approximately half oil-palm and half swamp forest. Rodents were trapped once monthly, and after the chiggers were removed, the rodents were marked by toe clipping and released at the site of capture. The chiggers were brought back to the laboratory for identification.

Table 8. Antigenic strains of isolates from animals trapped at Bukit Mendi oil-palm scheme.

Antigens	Animals	
	Number	%
Karp	10	27.0
Karp, TA 686	4	10.8
Karp, TA 763	12	32.4
Karp, TH 1817	1	2.7
Karp, TA 686, TA 763	4	10.8
Karp, TA 763, TH 1817	2	5.4
Karp, Gilliam	1	2.7
Karp, TH 1817, Gilliam	1	2.7
Gilliam, TC 586	2	5.4
Total	37	99.9

Table 9. Antigenic strains of isolates from animals trapped in different grids at Bukit Mendi oil-palm scheme.

Antigens	PHASE I (7)		PHASE III (FF)		PHASE III (YY)	
	Number	%	Number	%	Number	%
Karp	-	-	6	27.3	4	28.6
Karp, TA 686	-	-	3	13.6	1	7.1
Karp, TA 763	1	100	7	31.8	4	28.6
Karp, TH 1817	-	-	-	-	1	7.1
Karp, TA 686, TA 763	-	-	3	13.6	1	7.1
Karp, TA 763, TH 1817	-	-	1	4.5	1	7.1
Karp, Gilliam	-	-	-	-	1	7.1
Karp, TH 1817, Gilliam	-	-	1	4.5	-	-
Gilliam, TC 586	-	-	1	4.5	1	7.1
Total	1	100	22	99.8	14	99.8

Table 10. Antigenic strains of isolates from different animals trapped at Bukit Mendi oil-palm scheme.

Antigens	<u>R.tiomanicus</u> Number %	<u>R.argentiventer</u> Number %	<u>R.exulans</u> Number %	<u>Tupaia glis</u> Number %
Karp	8 25.8	1 25	- -	1 100
Karp, TA 686	4 12.9	- -	- -	- -
Karp, TA 763	10 32.3	2 50	- -	- -
Karp, TH 1817	1 3.2	- -	- -	- -
Karp, TA 686, TA 763	3 9.7	- -	1 100	- -
Karp, TA 763, TH 1817	2 6.5	- -	- -	- -
Karp, Gilliam	- -	1 25	- -	- -
Karp, TH 1817, Gilliam	1 3.2	- -	- -	- -
Gilliam, TC 586	2 6.5	- -	- -	- -
Total	31 100.1	4 100	1 100	1 100

Table 11. Identification of scrub typhus organisms in L.(L.) deliense and L.(L.) arenicola chiggers collected at Bukit Mendi oil-palm scheme in 1976/1977.

Month	<u>L.(L.) deliense</u>		<u>L.(L.) vivericola</u>	
	Pos/Total*	% Pos	Pos/Total	% Pos
Mar 76	4/90	4.4	0/0	0
Apr	1/91	1.1	0/0	0
May	20/445	4.5	0/40	0
Jun	-**	-	-	-
Jul	-	-	-	-
Aug	1/58	1.7	0/0	0
Sep	0/0	0	0/0	0
Oct	10/223	4.5	0/95	0
Nov	3/113	2.7	2/70	2.9
Dec	10/134	7.5	4/32	12.5
Jan 77	7/131	5.3	7/105	6.7
Feb	4/67	6.0	0/0	0
Mar	20/351	5.7	2/16	12.5
Total	80/1703	4.7	15/358	4.2

\* Pos/Total = Number of chiggers in which scrub typhus organisms were identified/total number of chiggers.

\*\* No samples were collected for identification.

Table 12. Antigenic strains of isolates from chiggers  
collected at Bukit Mendi oil-palm scheme.

Antigens	<u>L. (L.) deliense</u>		<u>L. (L.) vivericola</u>	
	Number	%	Number	%
Karp	58	72.5	13	86.7
Karp, Kato	2	2.5	-	-
Karp, TA 686	2	2.5	-	-
Karp, TA 716	1	1.25	-	-
Karp, TA 686, TA 716	1	1.25	-	-
Karp, TA 716, TA 763	1	1.25	-	-
Karp, TA 686, TA 716, TA 763	15	18.75	2	13.3
Total	80	100.0	15	100.0

During the early part of the study extensive surveys using the black plate technique were made to locate areas which contained chiggers. These surveys were confined to one grid within each Phase: Block 9 in Phase I and FF in Phase III. Findings immediately showed that Phase I chiggers were confined to the litter piles and could not be found in the grassy area between the trees. In contrast to the findings in Phase I, chiggers were found in both the grassy area and in the litter piles in Phase III. In each grid the areas where chiggers were found (mite islands) were marked and monthly collections using 10 black plates per location were made. Attempts were made to leave these areas undisturbed and only the chiggers collected from the 10 black plates were removed from the site.

Throughout the entire study, R. tiomanicus was the predominant species of rodent collected. Figure 6 presents the number of R. tiomanicus collected per month for all the study sites.

Figure 7 presents the number of L. (L.) deliense per R. tiomanicus collected from all study sites during the course of the study. In general, the number of L. (L.) deliense from Phase I was considerably less than that of Phase III. In Phase I, Block 9 continually produced a higher number of L. (L.) deliense than Block 7. The number of L. (L.) deliense approached zero within Block 7 throughout much of the study, with only one rise in number during November-December 1976. The number of L. (L.) deliense fluctuated considerably in the sites in Phase III throughout the study. It is possible that the more open area of Phase III is more greatly influenced by environmental factors than the area of Phase I that had a closed canopy.

Data from black plate collections that were made during the latter part of the study are presented in Figure 8.

Climatological data was collected throughout the study, and ground level relative humidity readings were made during part of the study. Evaluation of this data in comparison to chigger populations on rodents and from black plate collections are currently underway.

Within the oil-palm, vector chiggers can be found in 2 distinct microhabitats: under the litter piles and in the grass between the rows (Figure 9). Of these 2 microhabitats, only the grassy areas are important as an area where human might become infected. Workers within the area would not come into contact with the chiggers under the litter piles. On the other hand rodents live in the litter piles, and thus may become infected in an area which offers little risk to humans. Using the black plate collection method, chiggers were collected from the litter piles in both Phase I and Phase III throughout the entire year, although there was a reduced number during the dry periods.

Number of *Rattus tiomanicus* Trapped from Grids in Oil Palm at  
Bukit Mendi, Pahang, Peninsular Malaysia.

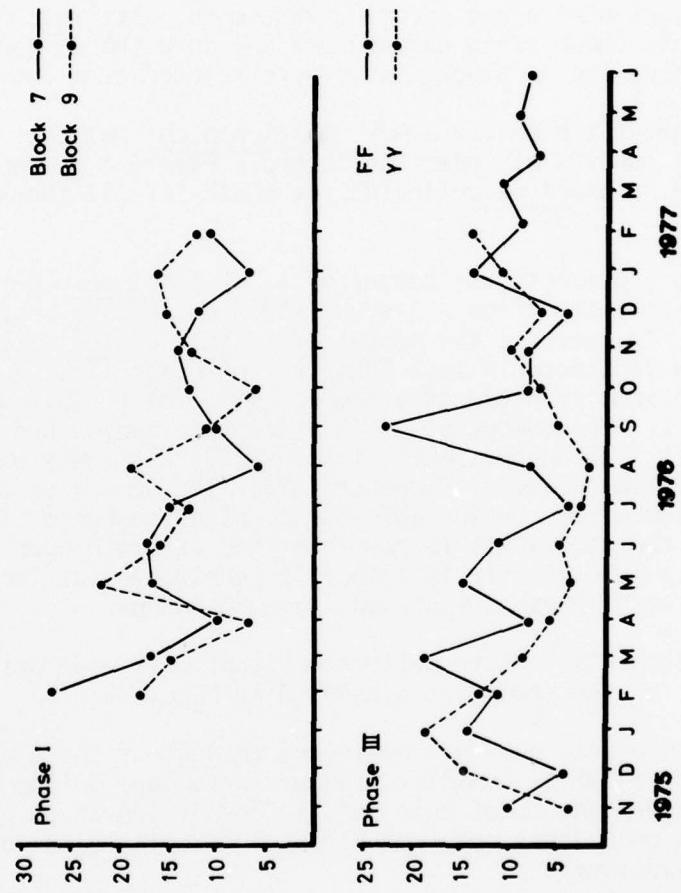


Figure 6

Number of Leptotrombiculum (L.) deliense per Rattus tiomanicus from Trapping  
Grids in Oil Palm at Bukit Mendi, Pahang, Peninsular Malaysia.

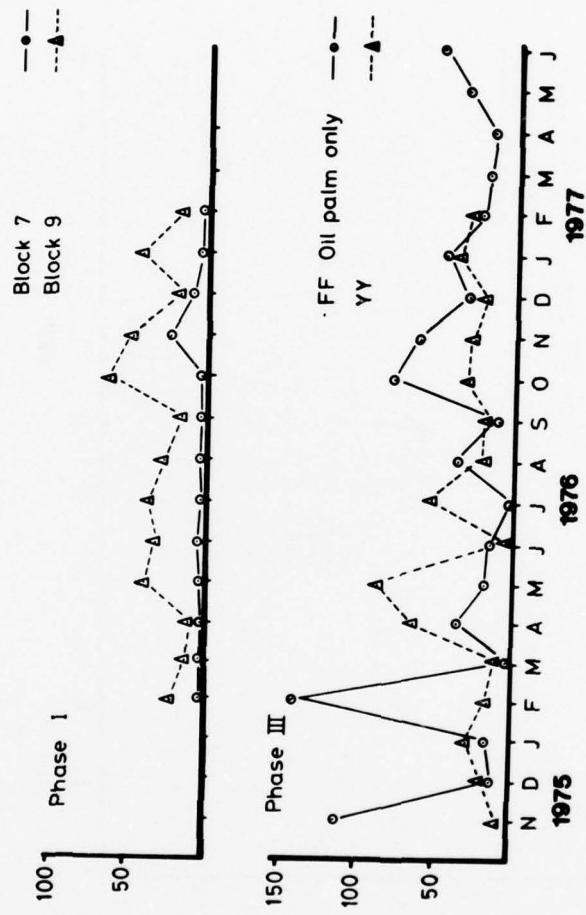


Figure 7

Number of *Leptotrombidium (L.) deliense* per Black Plate from  
Two Study Sites in Oil Palm at Bukit Mendi, Pahang,  
Peninsular Malaysia.

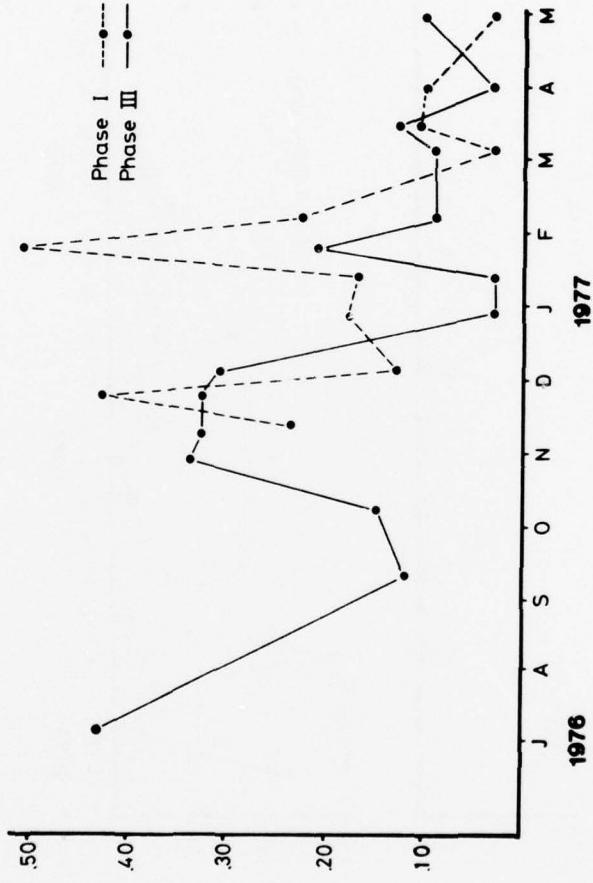


Figure 8

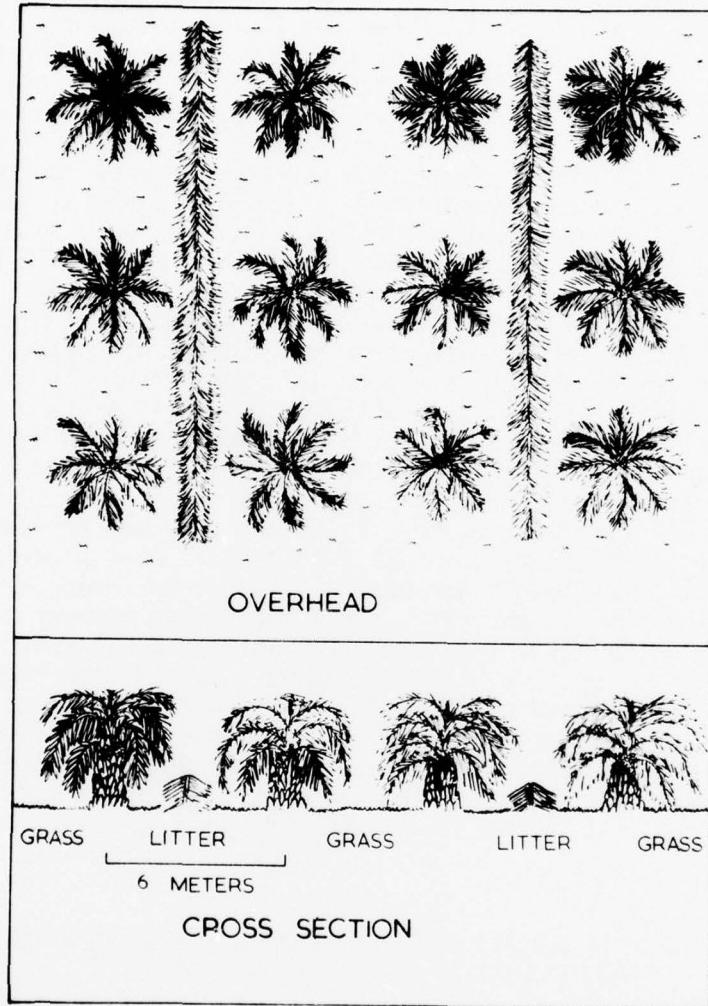


Figure 9

From the chigger collections at Bukit Mendi, as well as other recent studies, it seems that there may be an association between chigger populations and succession of vegetation in oil palm plantations. A specific sequence of events may explain the differences between Phase I and III within Bukit Mendi. Initially, vector chiggers are present within the forested habitats. As the forest is cut and burned, most of the vectors are completely eliminated, although small pockets of forest or swamps that contain the vectors and act as possible supplies of chiggers may remain. During the early stage of planting and growth of the oil-palm, rodents with their chiggers begin to reappear. Chigger populations may become established in isolated pockets, such as under logs or litter. However, chiggers are not able to become established in the open grassy areas between the oil-palm trees, as adequate moisture and shade is not available for their existence. Around 4-5 years after planting, the trees begin to shade over much of the grassy areas between the oil-palm. This shade, along with dead grass litter and fresh grass, is then a suitable habitat for vector establishment. It is at this time that the settlers are brought onto the scheme to begin working the oil-palm. As settlers harvest the fruit, they stack discarded palm fronds in the center of the rows of oil-palm trees. These palm fronds are stacked between every other row, and provide a good habitat for rodents and vector chiggers. Over a period of time the settlers clean their acreage, either by hoeing or by using weed killer. Eventually, the grassy areas between the trees - the ideal habitat in which the settler could come into contact with the vectors - is eliminated. By the time the oil-palm reaches 8-9 years of age, much of the area under the trees is cleared of vegetation that will support the vector chiggers. Although a large number of chiggers may continue to be present under the palm fronds litter, these chiggers are of little importance as a source of infection to humans.

#### ANTIBODIES TO SCRUB TYPHUS AND MURINE TYPHUS IN DOGS FROM SELANGOR, PENINSULAR MALAYSIA.

Although the importance of the dog in the epidemiology of rickettsioses, caused by the spotted fever group, has been well established (41, 45), its role in the ecology of other human rickettsial infections remains obscure. Numerous species of animals have been shown to be susceptible to infection with Rickettsia typhi, the causative agent of murine typhus, but little attention has been given to the dog (9, 101).

Dogs have been recorded as being hosts for Leptotrombidium akamushi and L. deliene (43), vector mites of scrub typhus. Serological studies by Alexander et al. (1) showed that dogs often became infected with Rickettsia tsutsugamushi in the endemic areas of Vietnam and provided evidence that they may serve as sentinels for scrub typhus.

In Peninsular Malaysia, recent studies have shown that scrub typhus and murine typhus are common causes of febrile illness (10, 11). This study presents the serological investigation of scrub typhus and murine typhus infections in dogs from different communities in Selangor, Peninsular Malaysia.

The communities, from which the serum samples were obtained, are shown in Figure 10. The rural areas are scattered throughout the state, whereas the metropolitan areas are located in and about the city of Kuala Lumpur. The metropolitan group is further subdivided into urban and suburban areas, the latter situated on the periphery of the Kuala Lumpur municipality.

Dogs from the kennels of the Society for Prevention of Cruelty to Animals (SPCA) were stray animals caught within the Kuala Lumpur area. All remaining dogs were privately owned and belonged to inhabitants of the respective study areas.

Blood specimens were collected by venipuncture and allowed to clot. Sera were taken and stored at -20°C prior to testing.

The sera were assayed using the IFA test (81). There were few modifications: (1) serial 2-fold dilutions of the sera were prepared in 0.01M phosphate buffered saline, pH 7.3, after the initial dilution of 1:25; (2) the 4 antigenic spots on the slides comprised of yolk sac preparations of Karp and Gilliam strains of *R. tsutsugamushi*, of *R. typhi* organisms, and of normal embryonated hens' eggs; and (3) the fluorescein-conjugated rabbit anti-dog globulin was prepared in our laboratory by methanol precipitation (28). The minimum titer accepted as significant was 1:50.

Scrub typhus antibodies were found principally in dogs from the rural areas (Table 13). Of the 97 dogs tested, 31 (32%) showed antibody titers to *R. tsutsugamushi*. It was interesting to note that there was a wide variance in scrub typhus antibody occurrence among the different localities, ranging from none at Bukit Tadom to over 80% of the sera tested from Bukit Lanjan.

Antibodies to murine typhus were detected in comparable numbers between the metropolitan Kuala Lumpur and the rural areas, 40% and 38% respectively. In the metropolitan group, the larger percentage of dogs having antibodies were from suburban areas of the city (Table 14). From the rural area, 32% of the dogs had demonstrable antibodies to both rickettsial organisms.

This study has demonstrated that natural infections of scrub typhus and murine typhus occur in dogs in Malaysia. The prevalence of scrub typhus antibodies was limited to the animals found in the rural areas. This finding correlates well with knowledge of the occurrence of scrub typhus, i.e. majority of cases occur in rural areas where patients come in contact with



Figure 10. Map of Selangor, Peninsular Malaysia showing location of communities from which specimens were collected.  
Settlements of Orang Asli (aborigines) are italicized.

Table 13. Prevalence of scrub typhus and murine typhus antibodies in dog sera collected from different communities in Selangor, Peninsular Malaysia.

Rural	Total	Scrub Typhus	Murine Typhus
Bukit Lanjan	16	13	8
Bukit Kemandul	15	3	6
Ulu Lui	11	2	2
Serendah	6	1	0
Bukit Tampoi	16	2	5
Bukit Tunggal	7	2	0
Kampung Bahagia	6	2	5
Bukit Tadom	12	0	9
Elmina Estate	8	6	2
	97	31 (32%)	37 (38%)
<hr/>			
<u>Metropolitan Kuala Lumpur</u>			
SPCA	32	0	9
Jalan Fletcher	7	0	1
Petaling Jaya	7	0	2
Ampang New Village	14	0	5
Salak South New Village	18	0	12
Jinjang North New Village	10	0	6
Selayang Baharu	9	0	4
	97	0 (0%)	39 (40%)

Table 14. Prevalence of murine typhus antibodies in dog sera collected from the metropolitan Kuala Lumpur, Peninsular Malaysia.

	Total	Murine Typhus
<u>Urban</u>		
SPCA	32	9
Jalan Fletcher	7	1
Petaling Jaya	7	2
	46	12 (26%)
<u>Suburban</u>		
Ampang New Village	14	5
Salak South New Village	18	12
Jinjang North New Village	10	6
Selayang Bahru	9	4
	51	27 (53%)

the infected chigger vectors. The variation in prevalence of antibody in sera collected from dogs at various rural sites may reflect the degree of infection in the respective areas. Indeed, previous studies by Mui et al. (73) showed a 75% prevalence of antibody to scrub typhus in small mammals collected at Bukit Lanjan. In the current study, 81% of sera collected from dogs at the same site demonstrated significant antibody titers to R. tsutsugamushi.

The prevalence of murine typhus antibodies was comparable in both rural and metropolitan areas. However, a further subdivision of the metropolitan area has revealed an interesting difference in the occurrence of antibodies in dogs (Table 14). Twenty six percent of the dogs from the urban area of Kuala Lumpur had demonstrable antibodies to R. typhi in contrast to 53% from the suburban areas. This discrepancy may be due to 2 factors: (1) more rigid rodent control within the city of Kuala Lumpur, depleting the reservoir of murine typhus rickettsiae; and (2) housing conditions in the suburban areas, conducive to harborage of rodents. The present findings correlate well with those of Alexander et al. (1) who serologically investigated military scout and tracker dogs in Vietnam for evidence of zoonotic infections. In these dogs which were worked and maintained in a rural type of habitat, the prevalence of antibody to scrub typhus was high. These workers suggested that the lack of antibody to flea-transmitted infections was due to the fact that military dogs were well provisioned and routinely treated with insecticides which readily controlled flea infestation.

While the distribution of dogs is world wide, they usually live in close association with man and are frequently exposed to the same disease organisms. In this laboratory, dogs experimentally infected with either scrub typhus or murine typhus organisms developed significant antibody titers (unpublished data). Under certain circumstances, the collection of specimens from dogs may be considerably easier than collection from either humans or rodents. Consequently, dogs could play important roles as sentinels in determining areas of potential risk to man.

#### L. (L.) VIVERICOLA. A POTENTIAL VECTOR OF SCRUB TYPHUS

As reported in the previous annual report (Walter Reed Army Institute of Research Annual Progress Report 1 July 1975 - 30 June 1976), a species of chigger taxonomically intermediate to L. (L.) deliense and L. (L.) arenicola was found to occur in several locations in the central part of West Malaysia. This species was referred to as near-L. (L.) arenicola, but since has been described as L. (L.) vivericola by Vercammen-Grandjean and Langston (95).

Of a total of 66 animals collected having L. (L.) vivericola, 57 (86.4%) were Tupaia glis, 7 (10.6%) were R. tiomanicus and 2 (3.0%) were R. argentiventer. Of the chiggers mounted and identified from the above animals, L. (L.) vivericola comprised 13.8, 5.5 and 3.7% respectively.

Of 358 L. (L.) vivericola chiggers collected at Bukit Mendi, 15 (4.2%) were found to be infected with R. tsutsugamushi. (See "Epidemiology of Scrub Typhus on a Malaysian Oil-Palm Development, 2. Rodent and Chigger Infections").

#### ANTIGENIC CHARACTERIZATION OF STRAINS OF R. TSUTSUGAMUSHI INFECTING CHIGGERS COLLECTED IN THAILAND.

In an effort to study strains of R. tsutsugamushi in areas geographically distant to central Peninsular Malaysia, where most of our field collections have been made, a cooperative study with the U.S. Army Component of the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand has been instigated. Unengorged chiggers are being collected by the AFRIMS personnel by the black plate collection technique throughout Thailand. R. tsutsugamushi infections in chiggers are being determined by the direct fluorescent antibody technique which is used simultaneously to antigenically characterize the strains.

#### EARLY DETECTION OF RICKETTSIA TSUTSUGAMUSHI IN PERIPHERAL MONOCYTE CULTURES DERIVED FROM EXPERIMENTALLY INFECTED MONKEYS AND DOGS.

The standard method of isolating rickettsial organisms is a long and laborious procedure. A rapid method of using tissue cultures of bone marrow cells and blood monocytes derived from infected guinea pigs to isolate Rickettsia rickettsii has been reported (12, 15). Primary monocyte cultures were also used to isolate spotted fever organisms from infected rhesus monkeys (15, 23). The sensitivity of this technique was similar to that of intraperitoneal inoculation of guinea pigs or plaque assay technique. Gambrill and Wisseman (37) described the growth of murine and epidemic typhus organisms in experimentally infected human macrophage cultures. The method for isolating Rickettsia tsutsugamushi organisms in laboratory mice often requires 3 months for completion. This report describes the isolation and cultivation of the scrub typhus rickettsiae in primary monocyte cultures from experimentally infected monkeys and dogs.

Silvered leaf monkeys, cynomolgus monkeys and dogs were inoculated intradermally with 0.05 ml of  $10^6$  mouse lethal dose

(MLD<sub>50</sub>) of *R. tsutsugamushi* (Karp strain). Three times weekly for 4 weeks, 20 ml of blood were collected aseptically from each animal using a 30 ml syringe coated with heparin (20,000 U/ml). Ten ml of warm 3% dextran (MW - 254,000) solution in normal saline were then added to the blood in the syringe. The syringe containing the blood-dextran mixture was placed in an upright position, with the needle end up, at room temperature for 60 min until the supernatant was clear. The plasma phase was then transferred into a sterile tube by gently pushing it through the bent needle while still holding the syringe in an upright position. Two ml of plasma were placed into each Leighton tube containing 9.5 X 35 mm coverslip. Tubes were incubated overnight at 35C, and then washed 2X with Hanks' balanced salt solution. Monkey cultures were maintained in Eagle's minimum essential medium with Earle's salts, supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine (400 mM), and 25 mM HEPES buffer, while the dog cultures were maintained in the same media with the exception that 20% heat-inactivated dog serum replaced the fetal bovine serum. Cultures were refed only when the media turned alkaline. Usually, the cultures did not require any change throughout the course of study. No antibiotics were used in the medium throughout the experiments.

Two coverslips were removed on each of 3 separate days, ranging from 9 to 16 days of culture. One coverslip was heat-fixed and stained with Giemsa technique, while the other was fixed in acetone for 10 min and stained with fluorescein conjugated anti-*R. tsutsugamushi* (Karp strain) rabbit serum. The test was controlled by using both conjugated normal rabbit serum and monocyte cultures prepared from uninfected control animals.

To determine the infectivity of the original blood samples and the fluid components in the monocyte cultures, 0.2 ml of each specimen was inoculated intraperitoneally into laboratory mice.

Sera were collected at each bleeding and tested by indirect immunofluorescence to determine the homologous antibody titers.

After 9-10 days of incubation, rickettsial organisms were identifiable by direct fluorescent antibody staining of peripheral monocyte cultures prepared 10 days post inoculation from all animals. Cultures were not stained before 9 days of incubation because of the limited number of monocyte cultures prepared at each bleeding. Monocytes derived from cynomolgus monkeys yielded the most consistent results, with the organisms being observed in cultures harvested through day 23 post infection. Dogs presented a more sporadic picture, but the rickettsiae were seen throughout the same time span. The cells from silvered leaf monkeys appeared to be much more fragile in that culture slides demonstrated fewer intact cells and more extracellular organisms, especially during the peak of infection.

Simultaneous observations of Giemsa-stained cultures demonstrated rickettsial-like organisms; however, the organisms were easily discernable only during the height of infection. At other times when the rickettsiae were present in decreased number, examination of Giemsa-stained cultures proved more difficult in that the organisms were often difficult to differentiate from other cellular components. Therefore, the use of direct immunofluorescence was the much preferred technique for detecting the rickettsial organisms. The rickettsiae did not stain with normal conjugate. Uninfected monocyte cultures did not show any organisms with either immunofluorescent or Giemsa staining.

Isolation of rickettsiae was accomplished from blood taken as early as day 6 post inoculation in case of silvered leaf monkeys but mostly day 9 post infection in cynomolgus monkeys and dogs. Presence of rickettsiae in the monocyte culture media generally corresponded to the time of rickettsial isolation from the blood.

Specific antibodies to Karp strain developed in all animals between days 13-20 post inoculation.

The success in detecting R. tsutsugamushi organisms in peripheral monocyte cultures derived from experimentally infected animals has prompted us to apply this methodology to the isolation of R. tsutsugamushi from human scrub typhus patients. Current study is in progress to evaluate the efficiency and sensitivity of this culture technique.

#### STUDIES OF CELL MEDIATED IMMUNITY IN RICKETTSIA TSUTSUGAMUSHI INFECTION

Preliminary studies in dogs and monkeys, experimentally infected with scrub typhus organisms, have demonstrated the feasibility of lymphocyte transformation assays for measuring the immune status. The major emphasis of our current work is on the development of adequate antigenic stimulants. The problem is two-fold: (1) the selection of the proper antigen to use in the test; and (2) the determination of whether that antigen is group-specific or strain-specific. Lymphocytes are currently being collected from scrub typhus patients in our study area in Pahang.

## IMMUNOGLOBULIN RESPONSES IN RICKETTSIA TSUTSUGAMUSHI INFECTIONS

This study has been delayed due to the priority of other studies.

### VIRULENCE AND ANTIGENIC PROPERTIES OF RICKETTSIA TSUTSUGAMUSHI IN A NATURALLY INFECTED LABORATORY COLONY OF LEPTOTROMBIDIUM (LEPTOTROMBIDIUM) ARENICOLA

Authors have commented on the variations in virulence encountered in naturally occurring strains of Rickettsia tsutsugamushi (54, 82). Rapmund et al. (76) noted that isolates of R. tsutsugamushi recovered from a colony of the vector, Leptotrombidium (Leptotrombidium) fletcheri, usually killed 10-20% of the inoculated mice at the second or third passage levels. However, a few isolates were more and others less lethal for mice at these passage levels. Traub et al. (94) studied a second L. (L.) fletcheri colony and found 18 of 19 isolates to be as lethal as strains regularly used in their laboratory. Few other data are available on the variations in virulence of R. tsutsugamushi organisms isolated from multiple generations of a colonized vector.

This laboratory has recently established a colony of L. (L.) arenicola which carries a mouse virulent strain of R. tsutsugamushi (79). This colony afforded us the opportunity to study variations in the virulence and antigenic markers of the organisms isolated over several generations of vectors.

The establishment of the colony and the methods of chigger rearing have been described (79). For testing, the larvae were allowed to individually engorge in the ear of a mouse. The mice were killed at 10-14 days post engorgement, and 0.2 ml of 20% liver-spleen suspension in sucrose phosphate glutamate buffer (7) was inoculated intraperitoneally (IP) into each of 6-8 mice. On the first day of signs of illness characterized by ruffled fur and ascites, 2 of these mice were killed to prepare passage material. The remaining mice were examined daily until death or 28 days post inoculation.

The virulence of the isolates was quantified by determining the mean death time of mice inoculated with the first passage of the isolate. From these numbers a mean and standard deviation was computed to compare isolates from different generations and different family lines. During the study of the early generations all mice which survived until 28 days post inoculation were challenged with  $10^3$  -  $10^4$  mouse IP lethal doses ( $LD_{50}$ ) of the highly virulent Karp strain to detect strains producing non-lethal immunizing infections.

For direct fluorescent antibody analysis mice were inoculated IP with 1.0 ml of a 1% glycogen solution in Hanks' BSS, and inoculated IP with 0.2 ml of a 20% rickettsial suspension 5 days later. The mice were killed when signs of infection were present, and the peritoneal cells were harvested by rinsing the peritoneal cavity with 5 ml of Hanks' BSS. Following concentration by centrifugation at 2,000 xg for 10 minutes at 4°C the pellet was resuspended in a minimum volume of Hanks' BSS. The direct assays were conducted by the method of Iida et al. (53) with the modification that dilutions of non-labelled heterologous sera were mixed with the monospecific conjugated sera to decrease the staining of the heterologous putative prototype strains (32).

Indirect fluorescent antibody assays were conducted by the method of Bozeman and Elisberg (8). Mice inoculated with representative isolates from each generation were treated with 2.5 mg/ml of chloramphenicol in the drinking water from days 3 through 24. They were bled at 28 days or later, and the sera tested for antibody specific to the 9 putative prototype strains of R. tsutsugamushi.

The number of isolates from each generation of mites and the mean death time of mice inoculated with these isolates are presented in Table 15. Little variation in virulence was seen among isolates or between generations. There was an increase in the mean death times of mice inoculated with isolates for the 7th and 8th generations.

No decreased virulence could be detected in isolates from 4 F<sub>4</sub> generation sibling mites which had 16 other siblings that did not transmit rickettsia to mice during feeding as larvae. Throughout the study there were occasional mice inoculated with spleen suspensions that did not succumb to the infection. However, when the mean death times of groups of mice inoculated with isolates that killed all the mice were compared with those from groups of mice having survivors, no significant difference could be detected ( $P > 0.05$ ). When family lines were studied no trend was discerned in times of death or numbers of survivors.

Table 16 lists the number of these survivors observed during the testing of each generation and line of mites. The differences seen from one generation to the next were not significant ( $P > 0.05$ ) and differences among sibling mites, when noted, could not be related to their sequence of birth, i.e. the survivors were not predominantly from isolations from early progeny or late progeny.

All isolates studied by direct fluorescent antibody methods contained antigens resembling those of the Karp and TA 763 putative prototype strains. Sera prepared from these isolates

Table 15. Mean death time of mice inoculated with isolates from an infected Leptotrombidium (L.) arenicola

Generation	No. Isolates	Mean Death Time (days)	Mean $\pm$ 2 S.D.
F <sub>1</sub>	1	10.9	- -
F <sub>2</sub>	12	12.2	11.3 - 13.1
F <sub>3</sub>	366	12.4	11.1 - 13.7
F <sub>4</sub>	148	12.6	11.2 - 14.0
F <sub>5</sub>	119	12.5	11.0 - 14.0
F <sub>6</sub>	338	12.4	11.1 - 13.7
F <sub>7</sub>	291	13.9	12.2 - 15.6
F <sub>8</sub>	252	13.0	11.9 - 14.1
F <sub>8</sub> <100% death	67	13.1	12.0 - 14.2
F <sub>8</sub> 100% deaths	185	12.9	11.8 - 14.0

Table 16. Number of surviving mice following IP inoculation of isolates from each of the generations of a Leptotrombidium (L.) arenicola colony. (Percent survivors shown in parenthesis).

Source of Isolate	Mice	
	No. Inoculated	Survivors
F <sub>1</sub>	8	0 (0)
F <sub>2</sub>	94	2 (2)
F <sub>3</sub>	1,807	55 (3)
F <sub>4</sub>	795	22 (3)
F <sub>5</sub>	779	33 (4)
F <sub>6</sub>	2,421	85 (4)
F <sub>7</sub>	1,734	43 (2)
F <sub>8</sub>	1,634	69 (4)
Total	9,272	309 (3)

in mice uniformly showed Karp and TA 763 antibody specificities.

No differences in the mean death time were detected from the 2nd through the 6th generations, but an increase was seen in the succeeding 2 generations. It is difficult to ascribe this difference to a change in characteristics of the rickettsia for the following reasons: (1) decrease virulence due to rearing of the chiggers in the laboratory would have been expected to have appeared during the first several generations; (2) a decrease in virulence of a few strains in each mite generation would have resulted in a gradually increasing death time with a concomitant increase in the standard deviation, whereas virtually all of the over 20 family lines studied in the F<sub>7</sub> and F<sub>8</sub> generations had increased mean death times; (3) based on our experience with the L. (L.) fletcheri colony strains a less virulent strain with an increased mean death time is also characterized by an increased number of mice surviving the infection. This was not observed during the study. Therefore, it was concluded that the increased mean death time was caused by factors other than a change in the virulence of the organisms during transovarial transmission.

Traub and Wisseman (93) in a comprehensive review of chigger borne rickettsiosis speculate that strains of varying virulence are maintained in the naturally infected vector with differing efficiencies. The rickettsia isolated from the L. (L.) fletcheri colony originally described by Rappmund et al. (76) has been and continues to be of mild virulence for ICR mice (unpublished data). The isolates from the L. (L.) arenicola colony are virulent to a degree comparable with the Karp strain, the most virulent of the classical strain for ICR mice. In the laboratory colonies both of the strain/vector combinations have usually produced 100% transovarial transmission rates, and for the preponderance of parent females, filial infection rates of 100%. Certainly any differences between the transmission of these 2 mildly and fully virulent strains were so slight as to be undetectable in our assay systems. The question of the efficiency of carriage of an avirulent strain can not be directly answered by our data, but it would be difficult to postulate any reason for decreased efficiency of carriage related to virulence.

Since the isolation technique was designed to recover avirulent as well as virulent strains a shift in virulence would not have affected the isolation rates. When a single negative larva occurred among 100% infected siblings, it was probably also infected, but when a series of negative offspring were produced, we feel they were not infected with R. tsutsugamushi rather than harboring a masked or avirulent infection which was not detected by the techniques used in this study.

Earlier work (80) had shown that approximately 80% of the ovaries of naturally infected adult chiggers contain rickettsial-like bodies that stain specifically in the direct fluorescent antibody test using labelled *R. tsutsugamushi* antisera. The 100% transovarial transmission rate measured in this colony indicated that 100% of the gravid females have organisms in their ovaries at some time during oviposition. Previous studies have shown (79) that some females will produce a series of progeny which will transmit the infection followed by a series which will not or vice-versa. There are distinct differences in the number of organisms seen in ovaries by direct fluorescent antibody methods. These differences are not related to changes in the antigenic makeup or virulence of the organism, because strains isolated from offspring of parent mites which did not produce 100% infected progeny were identical in these properties to strains isolated from progeny when all the siblings were infected. This indicated that the decreased filial infection rate was not a selection process or related to the virulence or antigenic makeup of the rickettsia but was a reflection of certain vector/rickettsial relationships which at times are less favorable for the multiplication of the rickettsia in the ovary of the trombiculid mite.

#### EXPERIMENTAL RICKETTSIA TSUTSUGAMUSHI INFECTION IN DOGS

The importance of the dog in the epidemiology of the spotted fever group rickettsiae has been well established. Recent studies with *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever, have demonstrated that the dog is a good model for the human disease (57).

The role of the dog in the ecology of scrub typhus is somewhat obscure. Blake et al. (3) briefly noted the early Japanese work where dogs were less susceptible to infectious material than monkeys. However, dogs have been recorded as being hosts for 2 species of vector mites, *Leptotrombidium akamushi* and *L. deliense* (43). Serological studies have indicated that dogs often became naturally infected with scrub typhus in the endemic areas of Vietnam (1). Recently, scrub typhus antibodies were found to be prevalent in dog sera, collected from rural areas of Selangor, Peninsular Malaysia.

The laboratory mouse has been primarily used as an animal model for the study of *Rickettsia tsutsugamushi* infection. It is well recognized that an intermediate animal model is required, and this study attempts to evaluate the dog as a potential candidate.

The dogs were inoculated with one of 2 R. tsutsugamushi strains, Karp or Gilliam, with different doses and by different routes. Temperatures were taken and physical signs were observed daily throughout the experiment. On designated days, blood was taken for hematological (hematocrit, hemoglobin, WBC, differential, thrombocytes), biochemical (SGPT, urea, albumin, total protein), serological (antibody by micro-IFA test), and rickettsemic (isolation in mice) studies.

Dogs inoculated intravenously (IV) with  $10^{5.5} - 10^{6.5}$  mouse infectious dose (MID<sub>50</sub>) of the Gilliam strain developed systemic signs including pyrexia, anorexia, vomiting, diarrhea, and severe weight loss. Some also showed CNS signs, and there were occasional deaths. Rickettsemia was rather long and pronounced.

In contrast, the animals inoculated intradermally (ID) developed eschars and regional lymphadenopathy, but systemic signs were minimal and no deaths were recorded. It appears that the occurrence of eschar and regional lymphadenopathy was dose-dependent, since only 50% of dogs infected with  $10^{3.5}$  MID<sub>50</sub> developed these local signs. Rickettsemia occurred but was observed about 5 days later and for shorter duration than in IV-inoculated dogs. Antibody responses were similar in dogs infected IV and ID (1:100 - 1:200).

Dogs inoculated ID with the Gilliam strain and subsequently challenged IV with the homologous strain developed no signs of disease. Antibody was not detectable either before or after challenge. Controls infected simultaneously developed the clinical signs as described above.

Dogs inoculated IV with approximately  $10^4$  mouse lethal dose (MLD<sub>50</sub>) of the Karp strain developed no clinical manifestations and did not die. Rickettsemia was demonstrated in these animals, as well as the development of low-titered (1:50) specific antibody.

There were no eschars formed in dogs infected ID with the Karp strain, but regional lymphadenopathy developed. However, when the dosage was decreased from  $10^{4.6}$  to  $10^{2.6}$  MLD<sub>50</sub>, only 50% of the dogs developed lymphadenopathy. There were no systemic signs, rickettsemia or deaths. There was an antibody response to the infection, and the titers were higher (1:100) than those found in IV-infected animals.

In general, dogs infected IV and ID with the Gilliam strain presented a more severe picture of infection than those infected with the Karp strain. Differences occurring from routes of inoculation were 2-fold: (1) in IV-inoculated dogs, systemic signs, including fever, were marked; and (2) although

rickettsemia occurs in both cases, it was demonstrated earlier and for a more prolonged time in IV-infected dogs.

EXPERIMENTAL INFECTION OF SILVERED LEAF AND CYNOMOLGUS MONKEYS  
WITH THE KARP, GILLIAM, AND KATO STRAINS OF  
RICKETTSIA TSUTSUGAMUSHI

Early works (44, 58, 60) have demonstrated the susceptibility of monkeys to the organisms of scrub typhus. Previous studies from this unit have used the silvered leaf monkeys (Presbytis cristatus) for experimental infections with different strains of Rickettsia tsutsugamushi (82, 97). However, the results (97) are difficult to interpret as each monkey was injected with multiple strains at different sites.

The need for a suitable intermediate animal host is well-recognized. Hence, the 3 "original" prototype strains of scrub typhus, Karp, Gilliam, and Kato, were used to inoculate the silvered leaf and the cynomolgus monkeys concurrently, so that comparisons could be made between the silvered leaf monkeys, which were used in all previous monkey studies in this unit, and the cynomolgus monkeys, which are the most readily available and the easiest to maintain.

Animals were infected intradermally (ID) with 0.05 ml of approximately  $10^4$  MLD<sub>50</sub> or MID<sub>50</sub> of the respective strain. Clinical signs, including temperature, were observed daily throughout the study. Blood was taken for the following studies: hematological (hematocrit, hemoglobin, WBC, differential); biochemical (SGPT, urea, albumin, total protein); serological (antibody by IFA); and rickettsemic (isolation in mice).

The monkeys inoculated with the Gilliam strain demonstrated overt clinical manifestations more readily than those with the Karp and the Kato strains. Both species of Gilliam-infected animals developed similar clinical signs with the exception of eschar formation. In the silvered leaf monkeys, the eschar formed in 20% more animals and persisted for a longer period of time than in the cynomolgus monkeys. There was also 60% mortality in the silvered leaf monkeys. There were 3 other silvered leaf monkey deaths, 2 infected with Karp and one with Kato, which occurred on days 46, 67, and 101. The one that died on day 46 had a Klebsiella abscess on day 32, but the definite causes of death in all 3 cases were unknown. The homologous antibody titers reached a much higher level in the cynomolgus (1:400 - 1:3200) than in the silvered leaf monkeys (1:100 - 1:200).

Eschar did not develop in the animals infected with either the Karp or the Kato strains, but regional lymphadenopathy

occurred in all cases. The Karp strain appeared more virulent than the Kato strain. The animals infected with the Karp strain developed fever as consistent as those infected with the Gilliam strain, while only 40% of the monkeys infected with the Kato strain had fever. Although cynomolgus monkeys did not die in either infection, Karp infection killed 40% of the silvered leaf monkeys, whereas no death occurred in monkeys infected with Kato. As in the case with Gilliam infection, the cynomolgus monkeys infected with the Karp and the Kato strains developed higher antibody titers (1:200 - 1:1600) than the silvered leaf monkeys (1:50 - 1:100).

Rickettsia was observed in both species of monkeys infected with all 3 strains of R. tsutsugamushi.

It appears that the Gilliam strain was the most virulent of the 3 strains in both species of monkeys. In most cases, the silvered leaf and the cynomolgus monkeys reacted similarly, but there were 2 significant differences: (1) death occurred in Gilliam-infected silvered leaf monkeys, while there were none in cynomolgus monkeys; and (2) the antibody responses in cynomolgus monkeys were greater than in the silvered leaf monkeys.

One must mention the difficulty in ascertaining the true effects of infection in silvered leaf monkeys. They are very fragile animals, in that up to 40-50% of the monkeys are lost during the conditioning period. In some groups, many monkeys were also lost after the conditioning period. Frequently in experiments, the uninfected controls die. The general thriftiness of these monkeys may have contributed to deaths of inoculated animals.

#### STUDIES OF RICKETTSIA TSUTSUGAMUSHI STRAINS PRESENT IN INFECTED LABORATORY-REARED LEPTOTROMBIDIUM (L.) ARENICOLA AND L. (L.) FLETCHERI CHIGGERS

##### 1. Natural infection of Silvered Leaf Monkeys

In any study dealing with a disease model, it is desirable to infect the animals by the natural route. A means of producing chigger-transmitted disease in controlled studies was considered important in the development of a reliable intermediate animal model in scrub typhus infection. Therefore, naturally-infected Leptotrombidium (L.) arenicola and L. (L.) fletcheri chiggers, both maintained in this unit (76, 79), were used to infect silvered leaf monkeys.

While the chiggers were allowed to feed within the capsule, the monkeys were maintained in a restraint chair (Figures 11 & 12). The design was based on a commercially available chair produced by Plas-Labs (Lansing, Michigan). This restraint chair allowed the monkey freedom of movement of hands and arms to feed

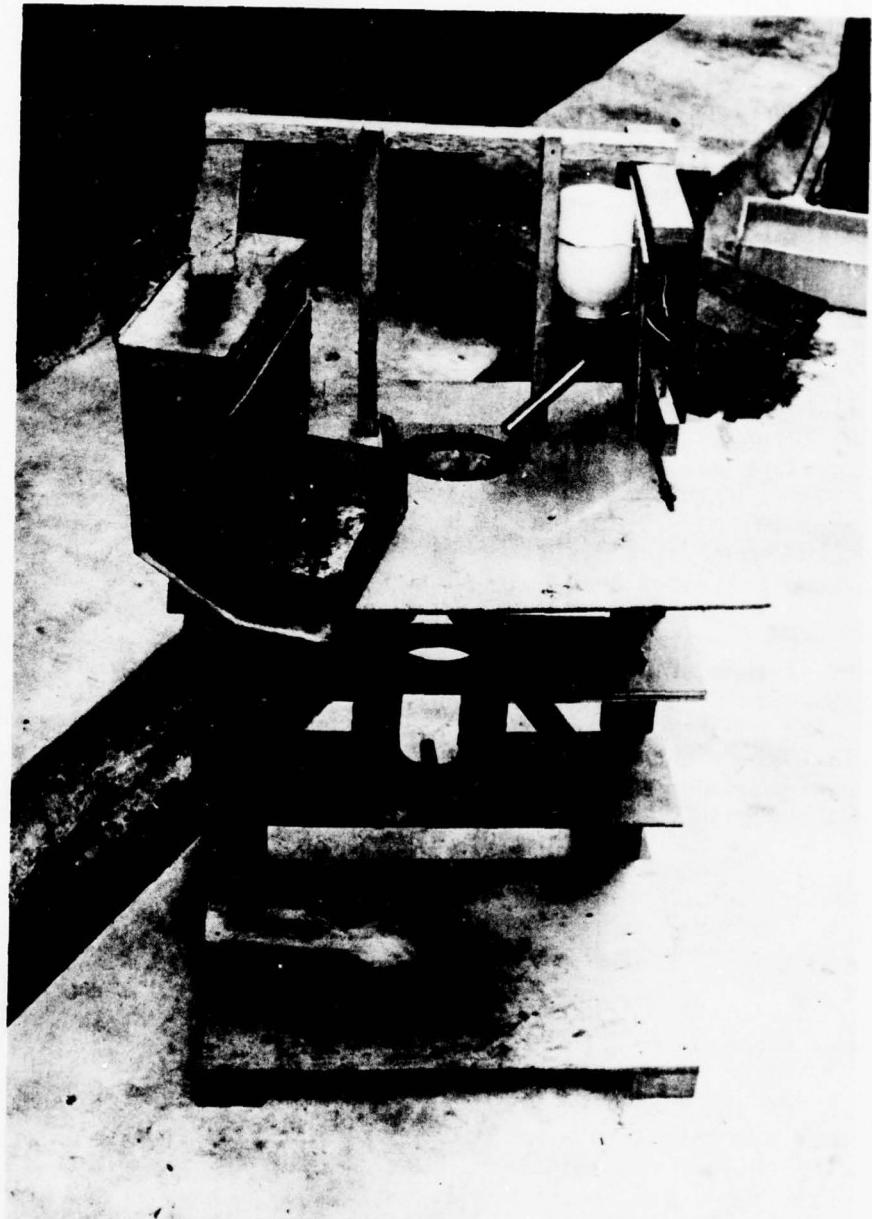


Figure 11

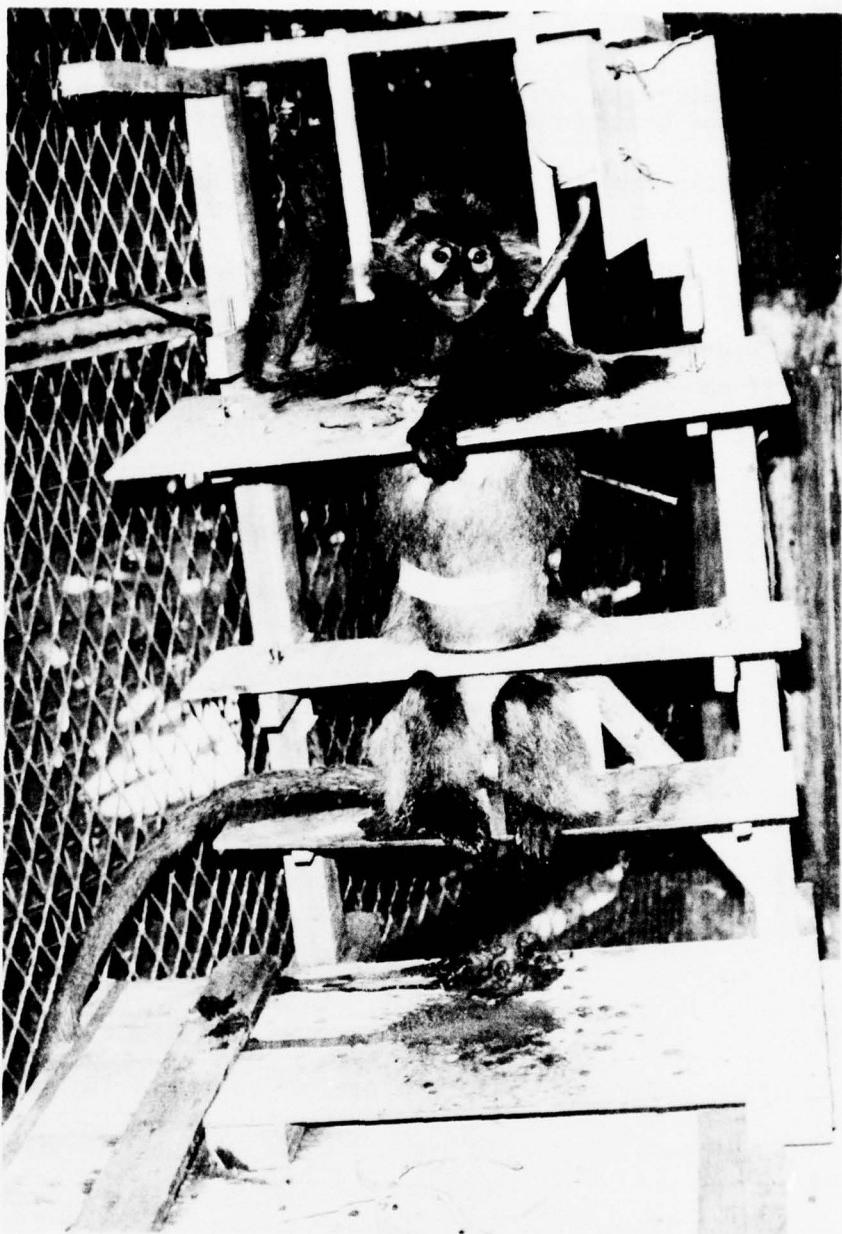


Figure 12

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and drink but did not allow the animal to reach its body below the chest region. The animals ate and drank in the chairs with no apparent difficulty and did not appear to be overly excited. The capsule could be opened and examined without immobilizing the monkeys, although, for close work such as counting chiggers, a light dose of sernylan was required for immobilization.

The capsule used for feeding the chiggers has recently been developed, allowing for application of larger number of chiggers and easy viewing of the engorging larvae (25). The capsule was constructed by cutting off the expanded portion of a disposable hypodermic syringe container. The resulting hole was smoothed of its rough edges, and glue (Bostick Universal Cement) was placed on the turned-under edges. This was then affixed to the medial surface of the thigh. The plastic top of the container was used as a cover for the capsule. A piece of moistened filter paper was placed in the cover, preventing dessication of the detached chiggers and allowing for ease of collection. Usually ten chiggers were placed in the capsules, and they were allowed to feed to repletion, which was normally 3 days.

The monkeys' temperatures were taken and physical signs observed daily throughout the study. On specified days, blood was drawn for hematological (hematocrit, hemoglobin, WBC, differential), biochemical (SGPT, urea, albumin, total protein), serological (antibody by IFA test), and rickettsemic (isolation in mice) studies.

Both chigger species were successfully fed on silvered leaf monkeys, and the responses in the animals were remarkably similar. With the exception of regional lymphadenopathy, there were no eschars, fevers, systemic signs or deaths. Sera collected one month after inoculation contained rather low fluorescent antibody titers (1:50). Rickettsemia and regional lymphadenopathy were more consistent with the animals infected with L. (L.) fletcheri than with L. (L.) arenicola chiggers.

It appears, therefore, that natural infection of the silvered leaf monkeys by the infected L. (L.) arenicola and L. (L.) fletcheri chiggers produced disappointing results as far as the development of any clinical signs.

## 2. "Needle"-Induced Infections in Mice and Primates

Due to the lack of overt clinical manifestations elicited by the naturally infected monkeys, seed suspensions of the strains existing in both colonies of infected chiggers were prepared from infected mouse tissues for further characterization of the strains in mice and monkeys.

Various dilutions of the suspension were inoculated intraperitoneally (IP) into mice (0.2 ml), and intradermally (ID) into monkeys (0.05 ml). The mice were observed for 28 days, and all survivors were challenged with  $10^3$  50% mouse lethal dose (MLD<sub>50</sub>) of the virulent Karp strain. The monkeys were bled periodically, and the following studies were performed: hematological (hematocrit, hemoglobin, WBC, differential); biochemical (SGPT, urea, albumin, total protein); serological (antibody by IFA test); and rickettsemic (isolation in mice). The monkeys' temperatures were taken and physical observations made daily throughout the study.

The seed suspensions were initially titrated in random-bred laboratory mice, and the following results were obtained: the Arenicola (A4822) strain had a MLD<sub>50</sub> of  $10^{-4.7}$  and MID<sub>50</sub> of  $10^{-5.1}$ ; the Fletcheri (A4899) strain was less virulent for mice in that the MLD<sub>50</sub> was less than  $10^{-1}$  and MID<sub>50</sub> was  $10^{-4.4}$ .

The Arenicola strain was titrated with doses of 4.7, 2.7, and 0.7 MLD<sub>50</sub> in silvered leaf and cynomolgus monkeys, and the only demonstrable clinical sign was regional lymphadenopathy in 1/3 silvered leaf and 2/3 cynomolgus monkeys receiving the highest dose. There were no fevers, eschars, systemic signs or deaths in either species. Rickettsiae were isolated from the peripheral blood of all silvered leaf monkeys, but from only 67% (6/9) of cynomolgus monkeys. However, all monkeys produced antibodies to the respective strains, and generally, the cynomolgus monkeys developed a higher titer than the silvered leaf monkeys. In fact, 22.2% (2/9) of the silvered leaf monkeys did not elicit any antibody response, even though scrub typhus organisms were isolated from their blood.

Three silvered leaf monkeys were infected with 3.2 MID<sub>50</sub> of the Fletcheri strain, and all showed varied responses. All developed rickettsemia, but only 2 had minimal antibody response (1:50). One animal died on the 24th day post inoculation, and when the spleen was harvested and inoculated into mice, rickettsiae were isolated from that tissue. Otherwise, all showed no signs of fever, eschar, lymphadenopathy, and systemic infection.

The Arenicola strain was more virulent for mice than the Fletcheri strain. However, the intradermal inoculation of both chigger strains into the monkeys resulted in poor clinical responses similar to those observed with natural infection.

## MAINTENANCE OF TROMBICULID MITE COLONIES

Two infected colonies of vector chiggers are maintained to study transmission of R. tsutsugamushi in the vector and provide a means of natural infection in experimental animals.

The infected colony of Leptotrombidium (L.) fletcheri, currently in its 26th generation, was collected from a talang field near Kampung Jendren, Peninsular Malaysia. The L. (L.) arenicola infected colony, in its 10th generation, was collected under vegetation along a sandy beach near Mersing, Peninsular Malaysia.

During the past year chigger colonies at the Walter Reed Institute of Research were lost due to low temperatures. Known infected adults from our L. (L.) fletcheri and L. (L.) arenicola colonies were shipped to Washington to restock the colonies. These were shipped after the adults had begun producing offspring, and the offspring had been tested for infectivity with the direct fluorescent antibody technique.

## FIELD STUDIES OF A SYSTEMIC ACARICIDE, DIMETHOATE, FOR THE CONTROL OF CHIGGERS (ACARINA: TROMBICULIDAE)

Chiggers have long been known to be a problem to both man and animals, either as vectors of disease or as causes of extreme irritation due to their mode of feeding. A few species of chiggers in Asia transmit the rickettsial disease, scrub typhus (Rickettsia tsutsugamushi), while quite a number of species throughout the world are known to attack man and cause an itching sensation and swelling at the site of their feeding.

Control of chiggers has been through the use of personal protective measures, cultural control (burning and clearing sites) and to a limited extent chemical control. Previous chemical control has relied on the spraying of acaricides in suspected habitats. Such operations are feasible only in limited situations. In Southeast Asia, the grassland habitat of the scrub typhus vector, Leptotrombidium (Leptotrombidium) fletcheri (=akamushi of Malaysia) (Womersley), could conceivably be sprayed, but not the densely forested areas where the vector, L. (L.) deliense (Walch), is often found. Similar situations occur throughout the world.

Systemic insecticide studies have proved successful against many blood-sucking arthropods, including fleas, lice and ticks (18, 19, 20, 27). The feeding habits of chiggers are somewhat unusual in comparison to other medically important arthropods in which systemic control has been effective. Chiggers primarily feed on digested tissue and not blood. Thus, in order for a

systemic control to be effective against chiggers, the acaricide must get into the tissue of the host.

Recently, Dohany et al. (24) studied the systemic effectiveness of 17 insecticides against chiggers. In these laboratory studies, of the 17 insecticides studied, only dimethoate proved effective in these laboratory tests. Upon demonstrating the effectiveness of dimethoate in these force feeding tests, Dohany et al. (24) conducted additional studies using dimethoate-treated baits. Baits with a concentration of as low as 0.1 percent were shown to be effective against chiggers fed on guinea pigs and cotton rats.

Dimethoate is a general usage insecticide distributed under the trade name CYGON (Trademark of the American Cyanamid Company). Dimethoate is recommended for usage in agriculture insect control, housefly control, forest insect control, ornamental insect control and as a plant systemic for the control of insects on certain fruits.

In systemic tests with dimethoate used against the Oriental rat flea, Clark (personal communication, 1974) found, when using essentially the same formulations of corn-milo mixture as used in this study, that the bait was acceptable at his lowest concentration (0.12 percent) but acceptability decreased at higher concentrations. In his tests, the rats were offered both treated and untreated baits.

The purpose of the current systemic acaricide studies at the IMR is to evaluate the efficacy of dimethoate as a control for chiggers in a field situation.

A rodent bait of 0.1 percent dimethoate formulated with a 1:1 mixture of cracked corn and milo was maintained in bait boxes. The bait boxes were 6 x 6 x 14 inches and were covered with a metal lid. Both ends are open to allow access by the rodents. The bait was changed weekly with the uneaten portion being removed to determine the amount of bait being consumed.

The bait was removed and rodents were trapped for 4 nights each month. The chiggers were collected from the rodents and the rodents were marked and released after trapping. The number and species of chiggers collected were determined.

Within the first study, an area of approximately 2.7 acres was treated with 50 bait stations located approximately 40 feet apart. The area was comprised of lalang (Imperata cylindrica) with a small area of scrub in the center. A similar control site was established within the same area but across the highway. The study in this habitat was terminated after 8 months when a fire eliminated most of the lalang in the dimethoate-treated area.

Following the burning of the lalang study site, the study was transferred to an oil-palm scheme. As an oil-palm scheme offers a good source and harborage for the rodents, it was thought that the rodents would not range very far and thus there would be less chance of immigration of rodents into the treated area.

The dimethoate treated oil-palm site consisted of approximately one acre of 6 year-old oil-palm. A total of 100 bait boxes were placed approximately 6 meters apart in a 60m x 60m grid. Traps placed in the same locations were used for the 4 nights of trapping each month. Rodents from a similar untreated control area were trapped at the same time as the treated area.

Twenty-two black plate collection sites were established within litter piles of the treated area and 15 in the untreated area with each site being sampled at least once a month. Ten 4 x 5 inch black formica plates were used at each site. The chiggers were collected in vials of alcohol for identification.

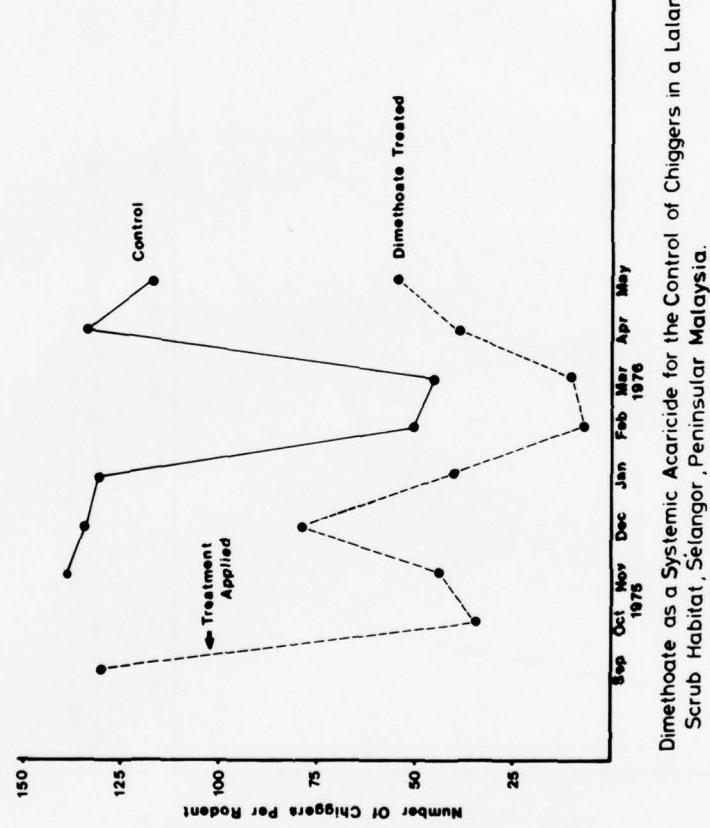
Figure 13 shows the number of chiggers taken from Rattus tiomanicus from the treated and control areas within the lalang study site. The dimethoate-treated line follows the same general patterns as that of the control.

Although a reduction in the number of chiggers per rodent was noted, complete control was not obtained due to the constant immigration of new rodents. As the lalang does not offer an abundance of food, rodents often range quite a distance. This caused a frequent reintroduction of new chiggers into the study site. This could be noted during the trapping by the collection of new un-marked rodents having large numbers of chiggers.

Although a large food source was available in the form of oil-palm fruit within the second study area, an equally good acceptance of the bait in comparison to the lalang study site was noted.

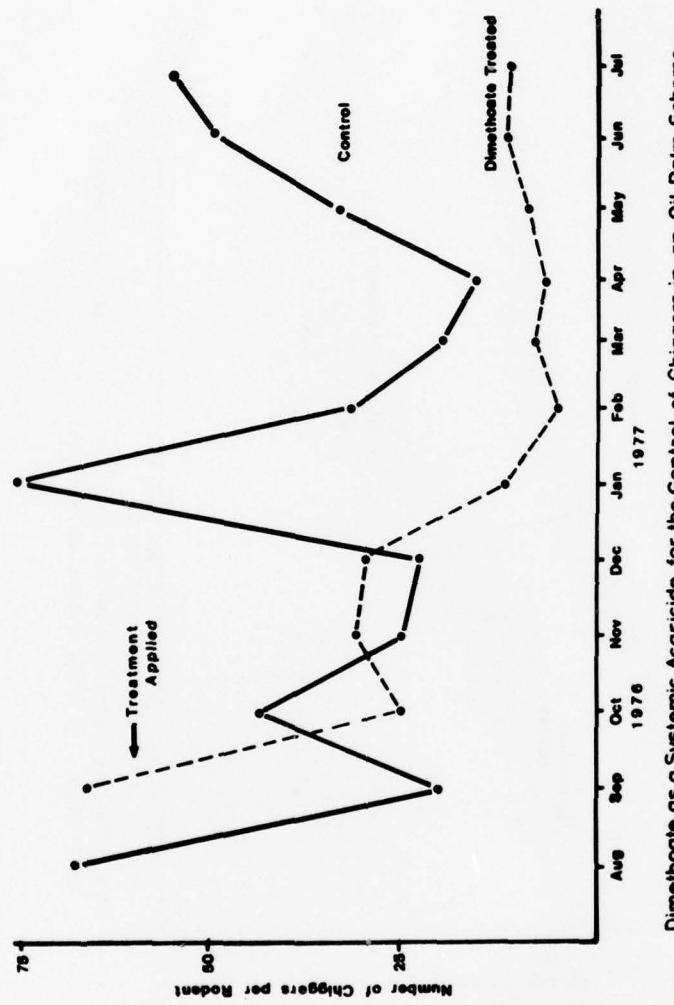
Figure 14 shows the number of chiggers per R. tiomanicus collected within the oil-palm habitat. A considerable amount of fluctuation can be noted in the first few months of the trial, but a reduction of the numbers occurred during the January collections and remained low throughout the study.

Figure 15 compares the number of chiggers per black plate within the treated and untreated oil-palm areas. During the initial 4 months of the study, the number of chiggers per black plate from the treated area tended to follow a similar pattern to that of the untreated area. As with the chiggers collected from rodents, the number of chiggers per black plate dropped considerably after December, with the level approaching zero during much of the remaining time.



Dimethoate as a Systemic Acaricide for the Control of Chiggers in a Lalang-Scrub Habitat, Selangor, Peninsular Malaysia.

Figure 13



Dimethoate as a Systemic Acaricide for the Control of Chiggers in an Oil Palm Scheme,  
Bukit Mendi, Pahang, Peninsular Malaysia.

Figure 14

Number of Leptotrombidium (L.) deliense per Black Plate from an  
Oil Palm Study Site Treated with Dimethoate Bait.

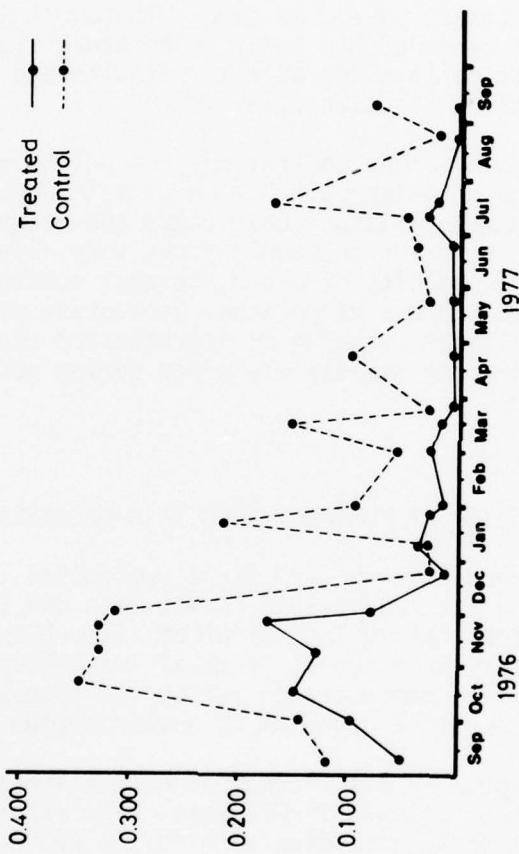


Figure 15

Many insects of medical importance in which systemic insecticides have been used as a control measure either attack its host in the adult stage or the adult stage is relatively short-lived. In the case of the trombiculid mite, only the larval stage feeds on a mammal host; thus, the soil dwelling adult continues to produce offspring that can attack animals. Control or total elimination of chiggers from a specific area using systemic acaricides can not be accomplished immediately as may be seen with other arthropods in which systemic control has been used (18, 19, 20). Reduction of the numbers of chiggers on the treated host may be noted rather quickly, but the overall population that would attack an animal would not be greatly reduced for a longer period of time. The adult life span of the chigger is considerably long. Laboratory studies have shown the adult to live for over 1 year with egg production throughout much of its life time.

Systemic acaricidal control may not be the ultimate answer for the control of chiggers. But in certain locations such as recreational parks, military base camps and frequent forest clearings, systemic control could prove very effective. However, in the majority of cases, chigger control will have to be relegated to the use of personal protective measures including repellants, wearing of impregnated clothing and washing with a cloth immediately after coming out of a suspected chigger habitat.

#### INVESTIGATION OF MURINE TYPHUS IN A MALAYSIAN VILLAGE

Fletcher and Lesslar (35) first recognised the existence of typhus in Malaysia, and in 1936, Lewthwaite and Savoor (62, 64, 65) demonstrated that murine, or urban (flea-borne) typhus was a separate entity from scrub, or rural (mite-borne) typhus. These workers also demonstrated rat to rat transmission by fleas of the causative organism of murine typhus (63).

Murine typhus is still recognised as a sporadic and uncommon cause of illness in Malaysia. The reported annual incidence of typhus (including both murine and scrub typhus) varied from 36-155 (mean 100) during the 7 years, 1968-1974 (Monthly Statistical Bulletin of West Malaysia, 1976). Although the clinical syndrome may be difficult to recognise, a diagnosis can be made by the Weil-Felix (WF) (35), complement fixation (CF) (46, 47), or indirect fluorescent antibody (IFA) (30) tests.

In a study of rickettsioses in Malaysia, Marchette (68) found murine typhus CF antibody in 10% of rats (Rattus rattus diardi) trapped from urban areas and in only one of 218 human sera obtained from a variety of sources.

In the course of an investigation of the causes of febrile illnesses in rural Malaysia (11), several cases of murine typhus were recognised in patients of Kuala Pilah hospital, Negri Sembilan. Two cases, in close succession, occurred in individuals who live only 3 houses apart in Senaling, a small village 2 miles south of Kuala Pilah. The first patient (H 6259), a 16 year old girl, was admitted to hospital on 20 May 1976 and the second, a 5 year old boy (H 6278), on 3 June 1976. Both diagnoses were confirmed by the WF and IFA tests.

In order to investigate the prevalence of the infection in Senaling, a serological survey was conducted of the residents, and of rats trapped in the village.

Sera were collected from 53 human volunteers, by house to house visiting, on 28 and 29 June 1976. A special effort was made to sample relatives and close neighbors of the two patients, though some of the sera were collected from residents of houses 100-200 meters away.

Rats were trapped using locally constructed basket traps (42). One hundred traps were given to 20 homeowners in the late afternoon on each of 2 days. They were to be set at night and collected early the next morning. Shortly after collection of the traps, the rats were anaesthetized with ether, combed for fleas and bled by cardiac puncture. The fleas were transported to the laboratory for identification.

All sera were examined for antibody to murine typhus by the IFA test (74, 81), in which the Wilmington strain of Rickettsia typhi (obtained from the Rickettsial Disease Section, Walter Reed Army Institute of Research) was used as antigen. The sera were diluted 2-fold from an initial dilution of 1/25. In addition, the human sera were examined for Proteus OX19 agglutinins (Wellcome Reagents, Beckenham, U.K.) by a microtiter technique (38). Sera obtained from a control group of 265 normal Malaysian soldiers were examined for antibody by the IFA test. No attempt was made to isolate the organism from humans, rats or fleas.

The serological results of the 2 index cases are shown in Table 17. The diagnosis was confirmed by a high IFA titer and a 4-fold rise in WF titer in H 6259. There was a significant rise in both IFA and WF titers in patient H 6278.

The IFA titers of the control and Senaling sera are shown in Table 18. Only 13 (5%) of 265 control sera titered 1/50 or more compared with 24/53 (45%) of the Senaling sera. Three (6%) of the Senaling sera titered 1/200 or more, and all 3 also had WF titers of 1/160 or more. One subject having an IFA titer of 1/800 and a WF titer of 1/160, was the 7 year old

Table 17. Serological results in patients  
H 6259 and H 6278.

	Date	FA	WF
H 6259	20 May	1/200	1/160
	25 May	1/400	1/640
	28 June	1/400	1/80
H 6278	3 June	1/25	<1/40
	5 June	1/1600	1/320
	28 June	1/400	1/80

FA = Fluorescent antibody titer to R. typhi.

WF = Weil-Felix Proteus OX19 titer.

Table 18. Murine typhus antibody in humans and rodents

FA titer	Number (Percent)		
	Malaysian Soldiers	Senaling Residents	Senaling Rats
<1/25	186 (70)	11* (21)	37 (53)
1/25	66 (25)	18 (34)	8 (12)
1/50	11 (4)	14 (26)	6 (9)
1/100	2 (1)	7 (13)	7 (10)
1/200	0	2** (4)	5 (7)
1/400	0	0	6 (9)
1/800	0	1*** (2)	0
Total	265 (100)	53 (100)	69 (100)

\* WF titer of 1/160 in 1 subject.

\*\*WF titers 1/320 and 1/640.

\*\*\*WF titers 1/160.

brother of patient H 6278, and lived in the same house. The father recalled no recent illnesses in the child. It is known from our records that this subject had been admitted to Kuala Pilah hospital a year previously (5 June 1975) with a febrile illness, and that paired sera collected at that time had IFA titers of <1/25 and WF titers of 1/40. Sera collected from the other 4 residents of the same house also had demonstrable antibody, including a 13 year old girl, whose IFA titer was 1/200 and WF 1/640. She denied any recent illnesses. The remaining subject with an IFA titer of 1/200, and WF of 1/320, was a 34 year old man, who admitted having had a short febrile illness, with headache, about one month prior to the survey.

Three relatives of patient H 6259, all of whom lived in the same house with the index case, had IFA titers of <1/25, 1/25 and 1/100 (all WF titers <1/160); and none admitted any illness.

Seventy-nine rats were collected from a total of 200 traps set during the 2 nights (40% trap rate). Of the trapped rats, 77 were Rattus exulans and 2 were Rattus rattus diardi. Sera were obtained from 69 of the 79 rats. The results of the IFA test on these sera are shown in Table 18. Altogether, 24 (35%) had titers of 1/50 or more, including 15/22 (68%) sera from rats trapped in three adjacent houses.

Forty of the 79 trapped rats were infested with a total of 102 Xenopsylla cheopis (flea index 1.3), with the number of fleas per infested rat varying from 1 to 8.

Senaling is a typical small village of West Malaysia with mostly ethnic Chinese inhabitants. The town itself consists of approximately 70 shophouses: coffeeshops, provision shops, rubber storage buildings and general ware shops. These shops are often connected in groups of 4 or 5, allowing for easy movement of rodents from one shop to another through the roofs and walls. The shophouses are usually constructed in such a manner that the shop area is in the front of the building and the living area is at the rear or upstairs.

Within the provision shops, food items are usually stored in sacks at the rear of the shop (Figure 16) and items for sale are placed in bins or open containers within the shop (Figure 17). Such practices provide sources of food and harborage for rodents, resulting in a large buildup of rodent numbers. This was noted in some of the provision shops. The traps were dispensed from 3:30 p.m. on the first day, with some shopowners setting their traps at this time. Before all the traps had been distributed (at 5:30 p.m.), 9 rats had been trapped. Most of these were from provision shops located adjacent to or near the two shophouses from which the first two cases of typhus were reported. The fact that such daytime trapping occurred indicated that there was a large, active population of rats.



Figure 16

Figure 17



The prevalence of antibody in the Senaling rats (35% at titers of 1/50 or more in the IFA test) was very high. Marchette (68) reported a prevalence of CF antibody in 10% of rats trapped from urban areas in West Malaysia.

Among the humans there was also evidence of a high prevalence of infection. In addition to the 2 index cases, 24 (45%) of the subjects studied had IFA titers  $\geq 1/50$ . The 3 subjects with IFA  $\geq 1/200$  also had significant WF titers, indicative of recent infections. Two of the 3 lived with one of the index cases, while the other lived in a house approximately 100 meters away. One of the 3 had had a febrile illness possibly attributable to murine typhus, though not clinically diagnosed as such. Forty five percent of the subjects with IFA titers of 1/50 or greater contrasts sharply with 5% of the controls. It is usual for ill people in communities such as Senaling to treat themselves, or to see general practitioners, who may prescribe antibiotics without a definitive diagnosis. Also, murine typhus is often relatively mild and self-limiting, and may often escape recognition. Unless Senaling was particularly unusual, it is likely that the infection is more common than is generally recognised.

In Senaling, there was a suggestion that a certain group of houses were particularly heavily infested with infected, flea-carrying rats, resulting in the several human infections. In the house where one index case occurred and in two adjacent houses, 68% of 22 rats had significant antibodies to murine typhus.

The district health authorities were notified of results of the survey within 10 days, and the villagers were given appropriate advice on the use of insecticides and traps.

#### EXPERIMENTAL INFECTION OF MIXED BREED DOGS WITH EHRLICHIA CANIS

Ehrlichia canis is the causative agent of canine ehrlichiosis, a widespread rickettsial disease of dogs, transmitted by the brown dog tick, Rhipicephalus sanguineus. The organism, which produces inclusions in the cytoplasm of infected leukocytes, was originally described in 1935 in Algeria (26). Infections have been reported from tropical and temperate areas in both Eastern and Western Hemispheres and parallel the distribution of the vector tick, R. sanguineus (34, 40, 49).

Canine ehrlichiosis may vary from a relatively mild febrile illness to a severe, chronic, fatal disease. In 1969 E. canis was shown to be the etiologic agent of a hemorrhagic disease of military dogs in Southeast Asia (51). Subsequent studies showed

that in certain breeds of dogs, particularly the Alsatian, a hemorrhagic syndrome, occurs approximately 60 or more days following *E. canis* infection and is associated with aplastic anemia (13, 14, 48). Although the hemorrhagic disease has been referred to by various names, "tropical canine pancytopenia" seemed the most descriptive and has been widely accepted in Southeast Asia (67, 85, 99).

In the 1960's canine ehrlichiosis with hemorrhagic manifestations accounted for the death of numerous military and privately owned dogs in Singapore (92, 100) and Malaysia (67); however, few studies of the disease in this country have been reported. The isolation of *E. canis* from a dog in Negri Sembilan, Peninsular Malaysia, afforded an opportunity to study the disease in experimentally infected, local mixed breed dogs.

The isolate of *E. canis* used in this study was obtained from a pet bull dog in Seremban, Negri Sembilan. The dog had been sick, and during routine hematological examination, organisms were seen in mononuclear cells in Giemsa-stained blood smears. Using a syringe wetted with heparin containing 20,000 U.S.P. units per ml, 15 ml. of blood were collected from the dog. The blood was transported to Kuala Lumpur on wet ice and was inoculated intravenously into a laboratory dog. The organism was maintained by serial passage in dogs.

The nine dogs in the study were mixed breed, adult dogs weighing 25 to 40 pounds. They were procured as puppies and maintained in individual cages in the laboratory. The dogs were fed a dry, commercially prepared, dog food and were provided water *ad lib*. Each dog was treated for intestinal parasites and given vaccinations for distemper, hepatitis and leptospirosis according to standard recommendations. With the exception of the dog inoculated with blood from the original case, each dog was inoculated intravenously with 10 ml of whole blood collected in ethylene diamine tetracetic acid (EDTA) from a dog acutely ill with the infection. Of seven infected dogs studied, four were inoculated with blood from the third passage and one each with blood from the original case, first passage and second passage. Two dogs were used as uninfected controls and were examined in the same manner as the infected dogs.

Following inoculation, each dog was examined daily and rectal temperatures were recorded. Thrice weekly during the first 106 days of the 220 day observation period, and weekly thereafter, blood was collected in EDTA for hematological examination which included determinations for hemoglobin, hematocrit, total white cell count, thrombocyte count and erythrocyte sedimentation rate (ESR), using standard laboratory procedures.

During the acute phase, blood smears were prepared three times a week from each dog, stained with Giemsa, and examined for inclusions of *E. canis*. Prior to inoculation and monthly thereafter, serum was collected from each dog and stored at -20°C until tested. For detection and titration of antibodies the indirect fluorescent antibody test described by Ristic et al. (77) was used. Sera were screened at a 1:10 dilution, and all sera with demonstrable antibody were serially diluted 2-fold and titrated.

Although some dogs had rectal temperatures of 103°F or greater as early as 9 days post inoculation, all dogs were febrile at 12 days. Pyrexia persisted for approximately 2 weeks. Thereafter the mean temperature returned to near normal.

The thrombocyte count dropped sharply from over 300,000/mm<sup>3</sup> at time of inoculation to below 40,000/mm<sup>3</sup> by day 12 (Figure 18). The mean thrombocyte count remained under 100,000/mm<sup>3</sup> throughout most of the experimental period. On numerous occasions thrombocyte counts of 5,000/mm<sup>3</sup> or less were recorded.

~~The hematocrit fell to below 30% by day 12 post inoculation, remained constant near 30% through day 72 post inoculation, and then gradually increased to above 40% at approximately 120 days post inoculation (Figure 19).~~ The lowest hematocrit recorded was one value of 10% on day 77 post inoculation in a dog which succumbed to the disease. Hemoglobin levels followed the same pattern as that of the hematocrit.

The white cell count dropped from a mean of 18,000/mm<sup>3</sup> on day of inoculation to a low of 6100/mm<sup>3</sup> on day 97 (Figure 20). Repetitive white cell counts of less than 5,000/mm<sup>3</sup> were recorded for 2 dogs 56-72 days post inoculation.

Erythrocyte sedimentation rates rose rapidly from a mean of 2.33 mm/hr at time of inoculation to 34.57 mm/hr at 12 days post inoculation (Figure 21). Six of the 7 dogs had an ESR of at least 40 mm/hr sometime during the experimental period. One dog maintained an ESR of 5 mm/hr or below except for day 19 when it reached 23 mm/hr.

One dog which died with severe hemorrhagic lesions at 78 days post inoculation had marked pancytopenia at time of death (Figure 22).

Weight loss of 5 to 7 pounds was noted in each dog with a gradual return to normal in surviving dogs. Swelling of prescapular and popliteal lymph nodes was observed as early as 3 weeks post inoculation in 3 dogs and persisted for several months. Corneal opacity was observed in 2 dogs. In one dog corneal opacity became evident 3 weeks post inoculation and persisted

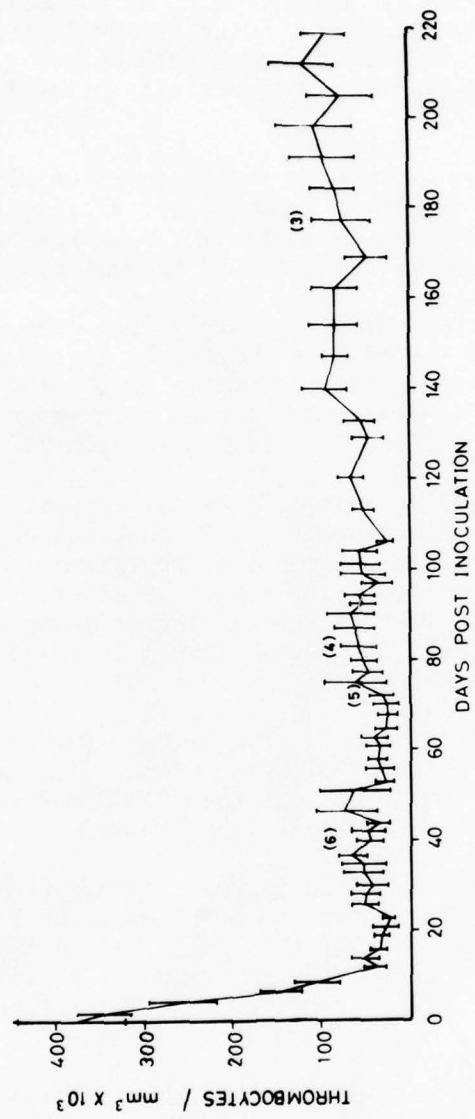


Figure 18. Mean thrombocyte counts of 7 dogs experimentally infected with *Ehrlichia canis*. The vertical bars represent the standard error of the mean. The number in parentheses indicates the number of dogs surviving.

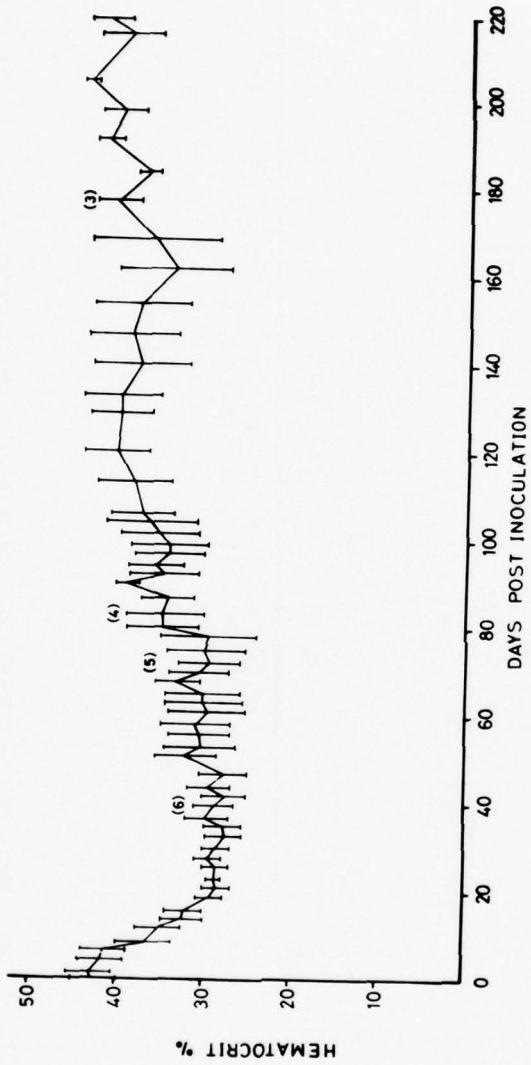


Figure 19. Mean hematocrit values of 7 dogs experimentally infected with *Ehrlichia canis*. The vertical bars represent the standard error of the mean. The number in parentheses indicates the number of dogs surviving.

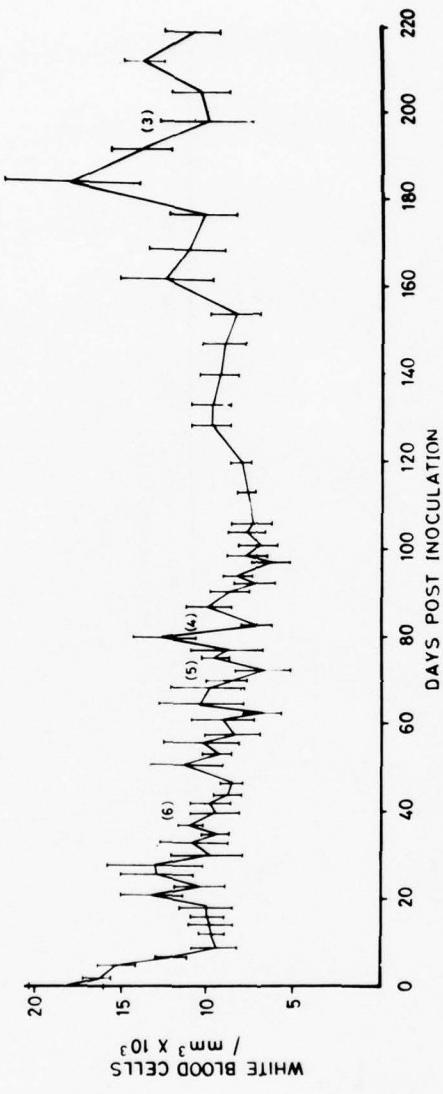


Figure 20. Mean white blood cell counts of 7 dogs experimentally infected with *Ehrlichia canis*. The vertical bars represent the standard error of the mean. The number in parentheses indicates the number of dogs surviving.

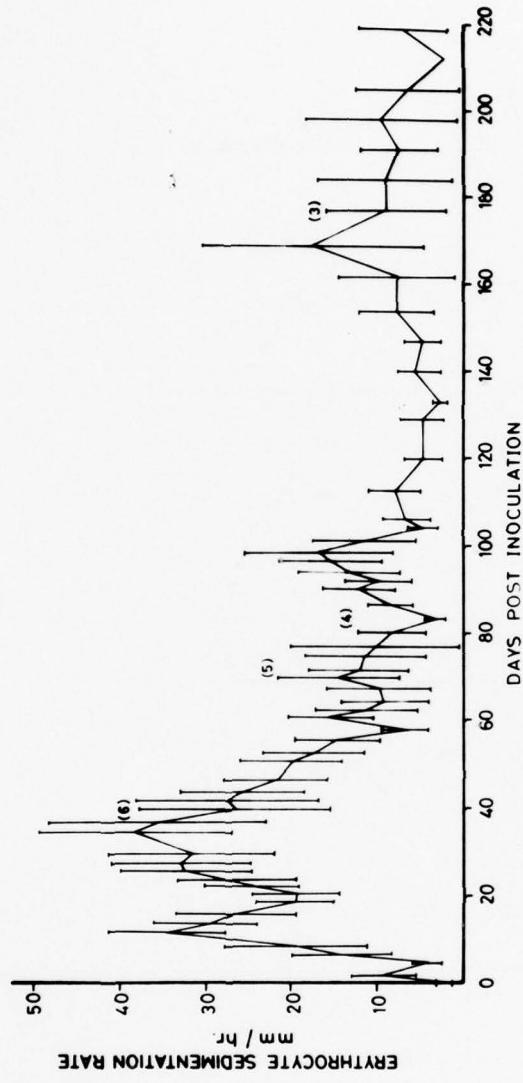


Figure 21. Mean erythrocyte sedimentation rates of 7 dogs experimentally infected with *Ehrlichia canis*. The vertical bars represent the standard error of the mean. The number in parentheses indicates the number of dogs surviving.

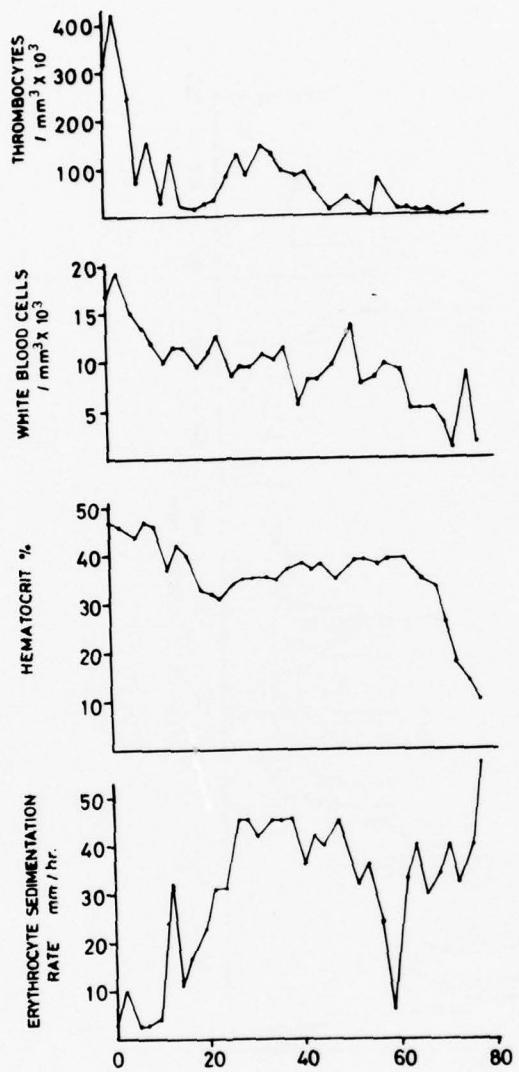


Figure 22. Thrombocyte count, white blood cell count, hematocrit and erythrocyte sedimentation rate of an experimentally infected dog that died 78 days post inoculation with marked pancytopenia.

throughout the observation period. In the other dog corneal opacity appeared 6 weeks post infection and persisted for 30 days.

Epistaxis, which is commonly seen in Alsatian dogs infected with E. canis, was not seen in our dogs; however, 2 dogs developed cutaneous petechial and ecchymotic hemorrhages at days 46 and 55 post inoculation. The hemorrhages were most noticeable around the shoulders, neck, flanks and abdomen. The hemorrhages on 1 dog disappeared within 3 weeks after they were first noticed. The other dog died approximately 3 weeks after the hemorrhages were first seen and the hemorrhages were still prominent at the time of death. A necropsy examination on this dog revealed a generalized hemorrhagic condition. Hemorrhages were seen in the skin and subcutaneous tissues, mammary glands, lymph nodes, lungs, heart (Figure 23), gastrointestinal tract, urinary bladder and pleura. The dog that recovered from the hemorrhagic condition later developed severe ascites and ventral abdominal and peripheral subcutaneous edema which was first noticed at day 120 post inoculation (Figure 24). This condition progressed until the dog died on day 170. At necropsy approximately 1500 ml of pale yellow, slightly opaque fluid were removed from the abdominal cavity.

In addition to the 2 dogs described above that died at 78 and 170 days post inoculation, one other dog died 38 days following infection. The only significant finding in this dog was ascites. A fourth dog became extremely emaciated and was killed 82 days post inoculation. Gross necropsy examination of the latter dog revealed no significant lesions.

The uninfected dogs showed no signs of disease and hematological values remained unchanged.

Serological examination of sera collected at monthly intervals from experimentally infected dogs revealed that antibodies to E. canis were present one month post inoculation and throughout the experimental period or until death. Antibody titers varied from 1:640 to 1:1280.

Morulae of E. canis in mononuclear cells in Giemsa stained blood smears were seen on occasion but were generally very difficult to find.

In conclusion, mixed breed dogs experimentally infected with E. canis developed clinical disease characterized by fever, anemia, weight loss, ascites, corneal opacity, hemorrhage, and death. Hematological examinations revealed pancytopenia and high erythrocyte sedimentation rates. The signs of disease were similar to those previously reported for natural and experimental infections in diverse geographical areas (6, 34, 50, 59, 86).



Figure 23. Hemorrhage in the heart of the dog  
that died 78 days post inoculation.



Figure 24. Severe ascites with ventral abdominal and peripheral edema in an experimentally infected dog which died at day 170 post inoculation. The picture was taken the day before death occurred.

The hematological findings (Figure 22) in the dog which died with numerous hemorrhagic lesions (Figure 23) 78 days post inoculation were similar to those frequently described in Alsatian dogs with tropical canine pancytopenia (48, 50, 52, 67, 99). Studies by Buhles et al. (13, 14) have clearly shown that aplastic anemia is important in the pathogenesis of the severe chronic disease, characterized by hemorrhage and death. Epistaxis, a common sign in dogs with the hemorrhagic form of disease, was not observed during this study.

The antigen used in the IFA test was prepared from an isolate of E. canis recovered from a dog in the U.S.A. In previously reported studies the test detected antibodies in dogs infected with isolates from Puerto Rico, the Virgin Islands, and Southeast Asia, suggesting antigenic similarity of isolates from various geographic locations (77). The high titers (1:640 and 1:1280) obtained during this study suggest antigenic similarity between the Malaysian isolate and isolates from various other geographic locations.

#### CYNOMOLGUS MONKEY BREEDING COLONY

In order to provide monkeys, not previously exposed to scrub typhus, the cynomolgus monkey breeding colony has been continued. Since the inception of the colony there have been 20 births recorded: 4 stillbirths, 4 deaths after birth, and 12 surviving infants, 8 of which have been weaned. From December 1976 to April 1977 no breeding occurred since the animals were housed individually while the colony was being moved.

Since instituting the procedure of placing lactating females in separate cages but still within the gang cage room, as previously described (Walter Reed Army Institute of Research Annual Progress Report, 1 July 1975 - 30 June 1976), there have been no deaths among nursing infants. In addition no fighting has occurred when females were reintroduced into the gang cage environment. Group two has continued to experience some difficulty with fighting, but replacement of the male has reduced the problem considerably. At present there are twenty females and two males in the colony. Six of the females are pregnant at the present time. Monthly weight and tooth eruption records are continuing to be maintained.

AN EPIZOOTIC OF MEASLES IN CAPTIVE SILVERED LEAF MONKEYS  
(PRESBYTIS CRISTATUS) IN MALAYSIA

The susceptibility of the rhesus monkey (Macaca mulatta) to measles virus infection has been known for many years and clinical signs of the disease have been described (4, 5, 75). In its natural forest habitat the rhesus monkey is considered to be free of measles but develop antibody to measles virus by the time they have been in a laboratory environment for a few weeks (70).

Cynomolgus monkeys (Macaca fascicularis) which are also relatively free of measles antibody in their natural habitat develop antibody rapidly after arriving in the laboratory (91, 102). Although antibody is produced, cynomolgus monkeys do not develop clinical signs of disease.

Other nonhuman primate species have not been studied as extensively as rhesus and cynomolgus monkeys in regards to their response to measles virus; however, Kalter et al. (56) reported that baboons (Papio sp.) were generally devoid of antibody to measles virus in their natural habitat but developed antibody as human contact increased. These workers reported the presence of measles antibody in chimpanzees (Pan sp.), orangutans (Pongo pygmaeus), gibbons (Hylobates agilis) and a vervet monkey (Cercopithecus aethiops) held at various facilities in the United States. Bhatt et al. (2) examined the sera of 170 bonnet macaques (Macaca radiata) and 195 langurs (Presbytis entellus) which were captured or shot in the forests of Shimoga District, Mysore State, India, and found none to be positive for measles antibody.

To our knowledge there have been no reports which demonstrate the natural occurrence of measles in the silvered leaf monkey (Presbytis cristatus), or the use of this monkey as a model for studying measles. This report describes a naturally occurring epizootic of measles in silvered leaf monkeys.

Thirty-one silvered leaf monkeys were observed during the epizootic. These animals were captured in a mangrove swamp area along the west coast of Peninsular Malaysia near the town of Kuala Selangor. The monkeys were caught by hand and were transported to our laboratory in individual cages the same day. The monkeys were placed in large cages with 3 or 4 animals per cage and were conditioned for 60 days during which time they were tuberculin tested every 2 weeks and wormed twice with levamisole (15 mg/kg). The monkeys also received daily injections of 300,000 units of penicillin for 3 days followed by daily injections of Trimethoprim (4 mg/kg) and Sulfadoxin (20 mg/kg) for 5 days. The monkeys were fed and maintained in a manner similar to that reported by Walker et al. (96). The animals had been conditioned in 2 separate groups and had been in the laboratory for 4 and 12 months respectively when the epizootic occurred.

Daily examinations and rectal temperature recordings were being made on 3 monkeys at the time the epizootic was first observed. On the same 3 monkeys thrice weekly hematological determinations for hemoglobin, hematocrit, total white blood cell counts and differential white blood cell count were being performed. These examinations were continued throughout the epizootic. The remainder of the animals were observed for clinical signs during the epizootic but temperatures and hematological examinations were not recorded.

Serum samples collected prior to the outbreak were available on all monkeys. Convalescent sera were collected from 23 animals at approximately 5 weeks following the initial observation of signs related to measles. All serum samples were stored at -20°C until tested for antibodies to measles virus by the complement fixation method using two-fold serial dilutions of the sera. A rise in titer to 1:16 or greater was considered as evidence of infection with measles virus. (The serological tests were done by LTC William H. Bancroft, MC, Department of Virus Diseases, Walter Reed Army Institute of Research).

A necropsy was performed on each animal that died during the epizootic. Tissues were collected in 10% neutral buffered formalin and sent to the Division of Pathology, Walter Reed Army Institute of Research, for histological examination.

Eight of the 31 monkeys died during the epizootic but their deaths could not be attributed to measles. Twenty-four monkeys were observed to have a rash or evidence of a rash (desquamating skin) during the period they were observed.

The rash was maculopapular in type and was distributed primarily on the ventral body surface although it was present to a lesser extent on the dorsal surface and the extremities. It was not observed on the palms of the hands or soles of the feet. The rash appeared to be most severe on the lower abdomen where the maculopapules were noted to coalesce in some cases. The rash persisted for 6 to 9 days, and when it disappeared, the skin began desquamating in small scales less than 1 millimeter in diameter to large sheets measuring up to approximately 1 centimeter in diameter (Figure 25). Desquamation continued for approximately 2 weeks after disappearance of the rash. Some animals had passed through the rash stage by the time they were observed, but desquamation of skin was considered as evidence that a rash had occurred.

A serous to mucopurulent nasal discharge and conjunctivitis were seen on occasion but were not consistent findings.



Figure 25. Picture of Silvered Leaf-monkey  
with measles.

The 3 monkeys on which thrice weekly hematological examinations as well as daily temperatures and observations were recorded developed a rash 8 days (2 monkeys) and 12 days (1 monkey) after recordings were begun. Leukopenia was noted 3 days after the rash started in 2 monkeys and 1 day prior to the rash in the third monkey. The leukopenia, which consisted of a 50% decrease in leucocytes occurred only for a single examination and the leucocyte counts returned to normal on the following examination for all 3 monkeys. One monkey did not develop a fever but of the other 2 monkeys one had a temperature of 103.0°F or above on 4 of the 8 days preceding the rash. The other monkey whose normal temperature was 98.6 to 100.6 had a temperature of 102.4°F on 2 of the 8 days preceding the rash.

Serological examination revealed that all 23 surviving monkeys had a pre-epizootic titer to measles virus of less than 1:4. Nineteen of the 23 monkeys developed titers ranging from 1:16 to 1:128.

No macroscopic lesions attributable to measles were found upon necropsy examination of animals that died during the epizootic. Histological examinations have not been completed.

The mode of transmission of measles virus to nonhuman primates has generally been suggested to be through human contact following capture of the monkeys (70, 91, 102). Shishido (91) further suggests that contact with the virus occurs before the monkeys arrive in the laboratory. This suggestion is based on the very early antibody conversion of monkeys following their arrival in the laboratory.

Silvered leaf monkeys live naturally in mangrove swamps and forest areas and do not come into close contact with humans. The monkeys in our study were placed in individual cages following capture and were transported straight to our laboratory for conditioning. Silvered leaf monkeys entering our laboratory would be expected to be free of measles antibody. This is supported by the absence of antibody in sera collected from the monkeys 4 to 12 months after arrival in the laboratory.

The method of introduction of measles virus into our laboratory is not known but 2 possibilities were considered. A technician who had daily contact with the monkeys stated that the children belonging to a close relative had had a rash a couple of weeks prior to the occurrence of the epizootic. In addition, the monkeys were housed on the ground floor in rooms separated from the outside only by wire mesh screen and on several occasions small children were observed to be peering at the monkeys which were only 1 to 2 feet away from the screen. It is possible that the technician or the children carried the virus to the monkeys.

The provision of a good nonhuman primate model for measles research requires the acquisition of uninfected monkeys in the wild and maintenance of the monkeys in the uninfected state in the laboratory. Nearly 500 silvered leaf monkeys have been conditioned in this laboratory over the past 10 years, and this is the first measles outbreak that has occurred. The monkeys were previously housed in an isolated building with very little human contact. The monkeys involved in this study, however, were the first groups housed in a different facility where there is potential for greater human contact. If silvered leaf monkeys could be procured in the same manner that we have described and taken directly to an isolated facility, they could probably be maintained in a measles-free state.

The suitability of an animal as a model for a human disease frequently depends upon the animal's clinical response to experimental infection. The clinical signs of measles in rhesus monkeys have been well described (4, 5, 75). The silvered leaf monkey develops good clinical signs of measles very similar to those seen in rhesus monkeys. The rash is maculopapular and is most noticeable on the ventral body surface. However, desquamation of the skin after the rash cleared seemed to be in larger sheets in the silvered leaf monkey than that reported for the rhesus monkey. The rash was seen in 24 of 31 silvered leaf monkeys during the epizootic. It is possible that those monkeys in which the rash was not observed had already passed through the rash and skin desquamation phases before the epizootic was discovered. The serous to mucopurulent nasal discharge and conjunctivitis seen in rhesus monkeys was seen in some silvered leaf monkeys. Reddening of the facial skin seen in the rhesus was not observed in silvered leaf monkeys; however, the pigmentation of the facial skin in the silvered leaf monkeys is dark grey to black in color and would probably mask the color change.

Following the epizootic reported in this paper a rash was noted in 5 of another group of 10 silvered leaf monkeys. The monkeys had been vaccinated for measles 10 days prior to the first observance of the rash. The rash did not appear to be as severe as that noted during the epizootic. The animals were being observed daily, and it was noted that coincident with the appearance of the rash, numerous small (0.2 to 0.5 mm), raised, circular, grayish-white lesions appeared on the oral mucosa including gums and tongue. These lesions resembled Koplick's spots although they did not have the typical purplish-red color surrounding them.

During the epizootic reported here in silvered leaf monkeys there were approximately 40 cynomolgus monkeys in our laboratory, housed in 2 rooms adjacent to the silvered leaf monkey rooms.

These cynomolgus monkeys did not develop clinical signs of measles during the outbreak. Yamanouchi et al. (102) has reported that cynomolgus monkeys infected with measles virus do not develop signs of disease. Potkay et al. (75) stated that other species in the Primate Quarantine Unit at N.I.H. have been observed for clinical signs of measles with negative results; these species included Cercopithecus aethiops, Saimiri sciurea, Macaca speciosa and Pan spp.

The silvered leaf monkey is probably free of measles antibody in its wild state and is capable of producing a good clinical response to measles virus; therefore, it could serve as a useful animal model in which to study measles infection. The limiting factor for the animal's use would be its fragility and limited availability. Walker et al (96) reported mortality rates of over 30% during the conditioning of silvered leaf monkeys and our experiences have been similar. Attempts to export the monkeys from the country of origin would likely result in an excessive mortality rate. In addition, the monkeys primarily inhabit the mangrove swamps along the coastal regions of Malaysia (96), and populations of these monkeys tend to be limited. Still, the silvered leaf monkey could serve as a useful animal model for measles virus research in limited numbers within the country of origin.

Since this epizootic our conditioning program for silvered leaf monkeys has been amended to include measles vaccination. Other research facilities and zoos maintaining this species, or even other langurs, should consider measles vaccination also.

#### MANAGEMENT OF TROPICAL LABORATORY ANIMAL RESOURCES

Responsibilities for the production and procurement of research animals are shared with the Institute for Medical Research. Approximately 2000 mice are weaned each week; of that number, USAMRU uses from 50 to 75%. Several other species are also bred in the facility.

Silvered leaf monkeys and cynomolgus monkeys are conditioned and held in our facility for experimental use. The current inventory is 34 silvered leaf monkeys and 60 cynomolgus monkeys. In addition to monkeys, random source dogs are conditioned and used in various studies. The current inventory is 37 dogs. Clinical data is being maintained on all animals undergoing conditioning and holding periods. Plans are underway to enter all data gathered into the new ADP system.

## ACQUISITION OF ADP EQUIPMENT

The systems design for a unit computer was undertaken and completed during the period December 1976 - April 1977 and a firm order placed for the necessary hardware and support systems. Detailed systems analysis is now virtually complete. Hardware delivery is expected in November 1977 and the whole package is expected to be operational by end-February 1978. It is proposed that this project be the subject of special reports in the next year.

Project 3M762770A802 TROPICAL MEDICINE

Task 00 Tropical Medicine

Work Unit 007 Field Studies of Rickettsioses and Other Tropical Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>b</sup> DA OC 6465	2. DATE OF SUMMARY <sup>b</sup> 77 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMRY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCY <sup>b</sup> U	6. WORK SECURITY <sup>b</sup> U	7. REGRADING <sup>b</sup> NA	8. DRSBN INSTR'N NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YBS <input type="checkbox"/> RD
10. NO./CODES <sup>b</sup> a. PRIMARY 62770A	PROGRAM ELEMENT PROJECT NUMBER 3M762770A802			TASK AREA NUMBER 00	WORK UNIT NUMBER 008	
b. CONTRIBUTING						
c. CDS/CMC/HG CARDS 114F						
11. TITLE (Proceed with Security Classification Code) (U) Tropical and Subtropical Diseases in Military Medicine						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>b</sup> 010100 Microbiology 002600 Biology						
13. START DATE 72 08	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House			
17. CONTRACT/GRAANT a. DATES/EFFECTIVE: NA b. NUMBER: c. TYPE: d. KIND OF AWARD:		EXPIRATION: b. PROFESSIONAL MAN YRS 77 9 FISCAL YEAR CURRENT 78 8	18. RESOURCES ESTIMATE b. FUNDS (in thousands) 503 550			
19. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research		20. PERFORMING ORGANIZATION NAME: US Army Medical Component, AFRIMS				
ADDRESS: Washington, DC 20012		ADDRESS: Bangkok, Thailand				
RESPONSIBLE INDIVIDUAL NAME: RAPMUND, G., COL TELEPHONE: 202-576-3551		PRINCIPAL INVESTIGATOR (FURNISH NAME IF U.S. Academic Institution) NAME: SEGAL, H. E., LTC TELEPHONE: 281-7776 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]				
21. GENERAL USE Foreign intelligence not considered.		ASSOCIATE INVESTIGATORS NAME: SCOTT, R. M., LTC NAME: HEMBREE, S. C., MAJ				
22. KEY WORDS (Proceed EACH with Security Classification Code) (U) Infectious Diseases; (U) Virus Diseases; (U) Bacterial Diseases; (U) Hepatitis; (U) Dengue; (U) Gonorrhea; (U) Enteropathogens						
23. TECHNICAL OBJECTIVE <sup>b</sup> , 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Proceed each with Security Classification Code.) 23. (U) To define the ecology and basic biology of causal agents of tropical diseases and to study environmental variables that may affect the performance of US servicemen in tropical areas. 24. (U) Routine diagnostic, epidemiological, serological, biochemical, microbiological, and entomological methods are being utilized. Field studies are emphasized and are supplemented by appropriate laboratory investigations. 25. (U) 76 10 - 77 09 Dengue, hepatitis and influenza were studied. Changes in dengue subtypes causing hemorrhagic fever were documented. Data suggested that this change in subtype might in part be responsible for the increased dengue incidence seen in Thailand. Recovery of dengue virus from platelets and leukocytes has increased isolation efficiency over 300 percent and may also help explain some features of the pathogenesis of the disease. Cell mediated immunity studies showed reductions in the percent and absolute prevalence of T lymphocytes in acute dengue infections. Hepatitis studies have indicated that hepatitis A (HAV) is less prevalent than hepatitis B (HBV) in US troops in Thailand. Investigations in urban and rural populations are delineating the prevalence of hepatitis B surface antigen (HBsAg), the incidence of HBV infections, the distribution of HBsAg subtypes, and the variables of transmission of HBV in children over one year of age. Continuing surveillance for influenza identified a A2(H3N2) outbreak. Bacterial studies indentified B Lactamase producing N. gonorrhoea in 8 percent of strains isolated from clinic patients. A high prevalence of enteropathogenic organisms were found in normal pregnant mothers at the time of delivery. Mosquito control potential was evaluated for 6 host pathogen pairs found in Thailand. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76-30 Sep 77.						
*Available to contractors upon ordinator's approval.						

DD FORM 1498  
1 MAR 68

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Work Unit 008 Tropical and Subtropical Diseases in Military Medicine (SEATO)

Investigators

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1. A Laboratory Study of Venereal Disease as Neisseria gonorrhoea in Male Patients over a 7 Month Period

OBJECTIVE: To investigate the status of N. gonorrhoea as a significant venereal disease in a population of males seen at the Royal Thai Army Hospital, Venereal Disease Clinic and the pattern of penicillin resistant organisms.

BACKGROUND: Neisseria gonorrhoea human infection has been followed in this laboratory for a number of years to periodically access the relative incidence in adults and to note patterns of antibiotic susceptibility found in Thailand. Previous studies did demonstrate a significant change in antibiotic susceptibility patterns (1). An increase in incidence of Neisseria betalactamase activity was suggested by routine diagnostic data.

METHODS: Specimens obtained were from urethral discharges in symptomatic males who were seen at the Royal Thai Army Hospital, Venereal Disease Clinic. Purulent discharges were streaked on Thayer Martin Agar, pH 7.2, supplemented with hemoglobin, Isovitalex and Vancomycin, Colistin, Mystatin. All incubations were at 35-37°C, in candle jars, and were observed at 24 and 48 hrs. Isolates identified as typical gram negative diplococci, oxidase positive and fermentating dextrose, sucrose and maltose were presumed N. gonorrhoea. Penicillin minimum inhibitory concentration tests by the agar plate dilution methods in 18 concentrations from 0.05 through 5.00 units per milliter were performed. In benicillinase producing N. gonorrhoea b-lactamase activity was accessed by an agar plate penicillin disk streptococcal permissive growth method.

RESULTS: Data are presented with pertinent case numbers in the table and is summarized as; the number of symptomatic patients seen, 171; the number of N. gonorrhoea isolates, 105; the number of patients seen more than once, 11; the number of isolates from same patient, 4; the number of PPNG isolates, 8; and the number of PPNG isolates from the same patient, 1.

Sixty-two percent of the symptomatic patients had gonorrhoea and fifty-six percent of those patients seen more than once had repeat infections. Eight percent of the N. gonorrhoea isolates were PPNG positive and of those one percent were repeat infections over the period studied. The pattern of penicillin susceptibility in MICs indicated that 55% of the specimens responded between 0.4 and 1.2 units/milliter.

It is apparent from the results that a periodic bacteriological survey on an annual basis is necessary in order to keep abreast

Table of Neisseria gonorrhoea infection case incidence and isolate penicillin susceptibility pattern.

January										March										June									
No.	Case No.	Date	N. gonorrhoea isolates	Pen. MIC	PPNG	No.	Case No.	Date	N. gonorrhoea isolates	Pen. MIC	PPNG	No.	Case No.	Date	N. gonorrhoea isolates	Pen. MIC	PPNG												
1	(1)	10/1	+	1.2	-	53	(53)	3/3	+	1.4	-	120	(121)	2/6	-	-	-	121	(123)	6/6	+	1.0	-	122	(123)	6/6	+	1.2	-
2	(2)	"	+	1.4	-	54	(54)	"	-	1.4	-	121	(123)	"	+	-	-	122	(124)	"	+	0.6	-	123	(124)	"	+	0.8	-
3	(3)	"	+	1.6	-	55	(55)	7/3	+	0.6	-	124	(125)	"	+	-	-	125	(126)	"	+	0.2	-	126	(127)	"	-	-	-
4	(4)	"	-	-	-	56	(56)	16/3	+	0.8	-	126	(127)	"	-	-	-	127	(128)	9/6	+	0.4	-	128	(129)	12/6	+	1.8	-
5	(5)	12/1	+	>40	+	58	(58)	"	-	0.8	-	129	(130)	"	-	-	-	130	(131)	"	+	0.1	-	131	(132)	"	+	0.4	-
6	(6)	"	+	1.2	-	59	(59)	"	-	1.0	-	132	(133)	20/6	-	-	-	133	(134)	"	+	1.4	-	134	(135)	"	-	-	-
7	(7)	"	+	0.8	-	60	(60)	21/3	+	0.8	-	135	(136)	"	-	-	-	136	(137)	22/6	+	1.0	-	137	(138)	2/8	+	-	-
8	(8)	"	+	1.2	-	61	(61)	"	-	0.2	-	138	(139)	"	-	-	-	139	(140)	30/6	-	-	-	140	(141)	"	+	0.4	-
9	(9)	13/1	+	0.4	-	62	(62)	23/3	+	1.0	-	141	(142)	7/7	+	1.4	-	142	(143)	"	-	-	-	143	(144)	"	-	-	-
10	(10)	17/1	+	2.5	-	63	(63)	"	-	1.0	-	144	(145)	"	-	-	-	145	(146)	"	-	-	-	146	(147)	"	-	-	-
11	(11)	24/1	-	-	-	64	(64)	"	-	0.8	-	147	(148)	14/7	+	1.8	-	148	(149)	18/7	+	1.4	-	149	(150)	"	+	2.5	-
12	(12)	"	+	>40	+	65	(65)	"	-	1.0	-	150	(150)	"	+	-	-	151	(151)	"	+	2.5	-	152	(152)	"	-	-	-
13	(13)	"	-	-	-	66	(66)	"	-	1.2	-	153	(153)	"	+	-	-	154	(154)	20/7	-	-	-	155	(155)	"	-	-	-
14	(14)	26/1	+	1.0	-	67	(67)	30/3	+	1.2	-	156	(156)	"	+	-	-	157	(157)	"	+	1.2	-	158	(159)	"	-	-	-
15	(15)	"	-	-	-	68	(68)	"	-	1.2	-	159	(159)	"	+	-	-	160	(160)	"	+	2.0	-	161	(161)	"	+	0.4	-
16	(16)	27/1	-	-	-	69	(69)	"	-	1.4	-	162	(162)	3/8	-	-	-	163	(163)	"	+	2.0	-	164	(164)	"	-	-	-
17	(17)	"	-	-	-	70	(70)	"	-	1.8	-	165	(165)	10/8	-	-	-	166	(166)	"	+	-	-	167	(167)	"	+	-	-
February										April										July									
No.	Case No.	Date	N. gonorrhoea isolates	Pen. MIC	PPNG	No.	Case No.	Date	N. gonorrhoea isolates	Pen. MIC	PPNG	No.	Case No.	Date	N. gonorrhoea isolates	Pen. MIC	PPNG												
18	(18)	3/2	+	1.0	-	71	(71)	"	-	1.4	-	168	(168)	17/8	+	0.6	-	169	(169)	"	-	-	-	170	(170)	22/8	+	0.4	-
19	(19)	"	+	0.4	-	72	(72)	"	-	0.2	-	171	(171)	"	+	-	-	172	(172)	"	-	-	-	173	(173)	"	-	-	-
20	(20)	"	+	0.6	-	73	(73)	31/3	-	-	-	174	(174)	"	-	-	-	175	(175)	"	-	-	-	176	(176)	"	-	-	-
21	(21)	"	+	1.8	-	74	(74)	"	-	-	-	177	(177)	"	-	-	-	178	(178)	"	-	-	-	179	(179)	"	-	-	-
22	(22)	8/2	+	1.6	-	75	(75)	7/4	-	-	-	180	(180)	"	-	-	-	181	(181)	"	-	-	-	182	(182)	"	-	-	-
23	(23)	"	+	1.8	-	76	(76)	"	-	-	-	183	(183)	11/4	+	3.0	+	184	(184)	18/7	+	1.4	-	185	(185)	"	+	2.5	-
24	(24)	"	+	1.2	-	77	(77)	"	-	0.2	-	186	(186)	"	-	-	-	187	(187)	23/4	+	3.5	+	188	(188)	"	+	-	-
25	(25)	"	+	1.2	-	78	(78)	"	-	4.0	+	189	(189)	"	-	-	-	190	(190)	"	-	-	-	191	(191)	"	-	-	-
26	(26)	"	-	-	-	79	(79)	"	-	1.4	-	192	(192)	"	-	-	-	193	(193)	"	-	-	-	194	(194)	"	-	-	-
27	(27)	"	-	-	-	80	(80)	"	-	2.0	-	195	(195)	"	-	-	-	196	(196)	"	-	-	-	197	(197)	"	-	-	-
28	(28)	9/2	+	1.6	-	81	(81)	"	-	-	-	198	(198)	14/7	+	-	-	199	(199)	18/7	+	1.4	-	200	(200)	2/8	+	2.5	-
29	(29)	"	+	1.0	-	82	(82)	"	-	-	-	201	(201)	10/8	+	-	-	202	(202)	14/8	+	1.8	-	203	(203)	18/8	+	1.4	-
30	(30)	10/2	+	0.4	-	83	(83)	11/4	+	3.0	+	204	(204)	18/8	+	-	-	205	(205)	20/8	+	2.5	-	206	(206)	24/8	+	2.5	-
31	(31)	"	-	-	-	84	(84)	"	-	0.05	-	207	(207)	"	-	-	-	208	(208)	2/9	+	-	-	209	(209)	6/9	+	-	-
32	(32)	"	-	-	-	85	(85)	"	-	-	-	210	(210)	10/9	+	-	-	211	(211)	14/9	+	-	-	212	(212)	18/9	+	-	-
33	(33)	"	-	-	-	86	(86)	"	-	-	-	213	(213)	"	-	-	-	214	(214)	2/10	+	-	-	215	(215)	6/10	+	-	-
34	(34)	2/20	-	-	-	87	(87)	23/4	+	3.5	+	216	(216)	10/10	+	-	-	217	(217)	14/10	+	-	-	218	(218)	18/10	+	-	-
35	(35)	"	-	-	-	88	(88)	"	-	2.5	-	219	(219)	"	-	-	-	220	(220)	2/11	+	-	-	221	(221)	6/11	+	-	-
36	(36)	"	+	1.0	-	89	(89)	"	-	1.6	-	222	(222)	"	-	-	-	223	(223)	10/11	+	-	-	224	(224)	14/11	+	-	-
37	(37)	14/2	+	1.0	-	90	(90)	"	-	3.5	+	225	(225)	"	-	-	-	226	(226)	18/11	+	-	-	227	(227)	2/12	+	-	-
38	(38)	"	-	-	-	91	(91)	"	-	-	-	228	(228)	10/12	+	-	-	229	(229)	14/12	+	-	-	230	(230)	18/12	+	-	-
39	(39)	"	+	1.0	-	92	(92)	2/5	+	5.0	+	231	(231)	10/13	+	-	-	232	(232)	14/13	+	-	-	233	(233)	18/13	+	-	-
40	(40)	"	+	0.1	-	93	(93)	"	-	-	-	234	(234)	2/14	+	-	-	235	(235)	10/14	+	-	-	236	(236)	14/14	+	-	-
41	(41)	16/2	-	-	-	94	(94)	"	-	4.0	+	237	(237)	18/15	+	-	-	238	(238)	2/15	+	-	-	239	(239)	10/15	+	-	-
42	(42)	"	-	-	-	95	(95)	9/5	+	1.2	-	240	(240)	18/16	+	2.0	-	241	(241)	2/16	+	-	-	242	(242)	10/16	+	-	-
43	(43)	"	+	1.0	-	96	(96)	"	-	0.2	-	243	(243)	18/17	+	-	-	244	(244)	2/17	+	-	-	245	(245)	10/17	+	-	-
44	(44)	"	-	-	-	97	(97)	"	-	1.2	-	246	(246)	2/18	+	-	-	247	(247)	10/18	+	-	-	248	(248)	14/18	+	-	-
45	(45)	17/2	+	1.0	-	98	(98)	"	-	0.4	-	249	(249)	18/19	+	-	-	250	(250)	2/19	+	-	-	251	(251)	10/19	+	-	-
46	(46)	"	+	1.0	-	99	(99)	"	-	1.2	-	252	(252)	2/20	+	-	-	253	(253)	10/20	+	-	-	254	(254)	14/20	+	-	-
47	(47)	23/2	-	-	-	100	(100)	"	-	1.0	-	255	(255)	18/21	+	-	-	256	(256)	2/21	+	-	-	257	(257)	10/21	+	-	-
48	(48)	24/2	-	-	-	101	(101)	16/5	+	2.0	-	258	(258)	18/22	+	-	-	259	(259)	2/22	+	-	-	260	(260)	10/22	+	-	-
49	(49)	"	-	-	-	102	(102)	18/5	+	0.4	-	261	(261)	18/23	+	-	-	262	(262)	2/23	+	-	-	263	(263)	10/23	+	-	-
50	(50)	"	-	-	-	103	(104)	"	-	0.1	-	264	(264)	2/24	+	-	-	265	(265)	10/24	+	-	-	266	(266)	14/24	+	-	-
51	(51)	"	+	2.0	-	104	(105)	"	-	1.2	-	267	(267)	18/25	+	-	-	268	(268)	2/25	+	-	-	269	(269)				

of the clinical and laboratory facets of N. gonorrhoea infections in Thailand in order to provide information for appropriate treatment and prevention.

2. Workload Data Base of Clinical Division Support in the Department of Bacteriology and Clinical Laboratory Science

OBJECTIVE: To assemble a data base of support workload in order to evaluate management efficiency relative to protocol and publication success at both the departmental and inter-departmental areas of research operations.

BACKGROUND: Research investigations in infectious diseases demands certain aspects of subject data that are compatible with clinical laboratory medicine systems.

The Department of Bacteriology and Clinical Laboratory Science was organized during August 1977 with the objective of identifying its research mission in the investigation of infectious diseases on departmental as well as on interdepartmental collaborative levels.

The Department is a research department which will initiate and maintain studies in infectious diseases of bacterial, fungal, and parasitological origin and or those clinical studies in chemistry and microscopy which are associated or predispose to infectious diseases. The clinical division is a research support division. All data produced by the department are to be identified in protocol and publication as a prerequisite to performance.

The departmental reorganization and emphasis on it having a principle research mission demanded the establishment of a support workload data base.

METHODS: Quarterly workload data in terms of number of tests cultures etc. were collected from laboratory records. Data were collated to US Component departments as Virology, Veterinary Medicine, Medical Entomology, Medicine, Bacteriology, Immunobiology, and Epidemiology. Two miscellaneous sections, that of other tests not occurring in great frequency, and other departments not US component divisions were utilized as data partitions. No analysis of work time elements of workload was attempted.

Work Load Table, FY 77

Clinical Chemistry

		Others		
Seroology	Virus (Oct-Dec '76) (Jan-Mar '77) (Apr-Jun '77) (Jul-Sep '77)	1 15		1
Urine	Vaccines			
Varietal	Other (Oct-Dec '76) (Jan-Mar '77) (Apr-Jun '77) (Jul-Sep '77)			
Urethritis	Bacilli			
Urinary tract	Eye/ear			
Blood	pus/effud			
CSP	Culture			
CSF	Vet-Med. (Oct-Dec '76) (Jan-Mar '77) (Apr-Jun '77) (Jul-Sep '77)	1 1 1 1		1
Eye	Virus (Oct-Dec '76) (Jan-Mar '77) (Apr-Jun '77) (Jul-Sep '77)	25 25 25 25		22
Eye/ear	Other (Oct-Dec '76) (Jan-Mar '77) (Apr-Jun '77) (Jul-Sep '77)	25 25 25 25		22
Eye/ear	Virus (Oct-Dec '76) (Jan-Mar '77) (Apr-Jun '77) (Jul-Sep '77)	1 1 1 1		1
Hair	Virus (Oct-Dec '76) (Jan-Mar '77) (Apr-Jun '77) (Jul-Sep '77)	1 1 1 1		1
Hair (cont'd)	Virus (Oct-Dec '76) (Jan-Mar '77) (Apr-Jun '77) (Jul-Sep '77)	1 1 1 1		1

Clinical Microscopy

	CBC	Platelets Count	Urticaria	GMPD	Other**
Virus (Oct-Dec '76)	261	234	7	214	111
(Jan-Mar '77)	266	245	18	316	60
(Apr-Jun '77)	359	337	18	-	23
(Jul-Sep '77)	617	268	2	-	12
Vert. - Med. (Oct-Dec '76)	110	-	-	-	-
(Jan-Mar '77)	157	-	-	-	-
(Apr-Jun '77)	90	-	-	-	-
(Jul-Sep '77)	-	-	-	-	-
Medication (Oct-Dec '76)	4	2	7	0	-
(Jan-Mar '77)	1	1	5	0	-
(Apr-Jun '77)	6	6	10	1	1
(Jul-Sep '77)	5	3	3	-	6
Bact. (Oct-Dec '76)	3	-	3	-	-
(Jan-Mar '77)	10	2	0	2	-
(Apr-Jun '77)	22	5	0	2	6
(Jul-Sep '77)	15	5	1	-	11
Immun. (Oct-Dec '76)	6	1	1	-	-
(Jan-Mar '77)	12	3	4	-	3
(Apr-Jun '77)	6	2	2	-	8
(Jul-Sep '77)	47	2	2	-	12
Epid. (Apr-Jun '77)	174	-	1	-	-
Other ** (Oct-Dec '76)	16	1	6	40	36
(Jan-Mar '77)	16	1	5	23	63
(Apr-Jun '77)	16	5	7	24	77
(Jul-Sep '77)	21	9	7	32	86

3

Chitosan Semidilute

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	Pyridostigmine	Sulfamethadiazine
Medicine (Oct-Dec 76)	30	58
Medicine (Apr-Jun 77)		85

<sup>6</sup> Laboratory personnel, Thai Component, Royal Thai Army Hosp., Navy Hosp., Children Hosp., Rajvithi Hosp., Prabuddhabat Hosp., Bangkia Hosp., Peace Corps, US Embassy.

RESULTS: Workload counts for the Clinical Bacteriology, Clinical Chemistry, Clinical Microscopy and Special Chemistry Section are presented as a table. The workload covers fiscal year 1977. Standard terminology and abbreviation of laboratory tests is used.

The data base indicates that production of the Department's Clinical Division is in fact supporting research efforts on both departmental and interdepartmental levels. The base does not comprehensively identify the success of such efforts in measurements of efficiency and design of protocols or publications. Further investigation as an ongoing subject is necessary as a matter of historical record, management experience, and design efficiency in the operation of overseas military medicine research activities.

3. Prepartum Rectal Bacterial Flora; Phra Mongkutkao Hospital, Bangkok, Thailand

OBJECTIVE: To define the rectal bacterial flora of mothers prior to immediate delivery at Phra Mongkutkao Hospital.

BACKGROUND: A previous study on neonatal diarrhea at the nursery of the Phra Mongkutkao Hospital was performed and demonstrated Escherichia coli of several serotypes colonizing neonatal gastroenteritis symptomatic infants and over 20% of the asymptomatic nursery staff (2). A follow-up, random prepartum, study during August 1977 of mothers delivering infants in this hospital was performed to assess the epidemiologic importance of mother to infant transmission.

METHODS: Patients were normal healthy mothers with no recent history or symptoms of diarrhea or gastroenteritis. Specimens were obtained upon admission to the delivery room prior to a preparatory enema. Swabs were transported in Cary-Blair media, plated on routine enteric media, and incubated for 24 hours at 37°C. Identification techniques utilizing standard enteric methodology were directed towards the enteric organisms of medical importance as Salmonella, Shigella, E. coli, and the Vibrios.

RESULTS: Bacteriologically significant isolates (50) of a total (103) isolates were identified.

### Enteropathogenic Isolates

<u>Salmonella</u>	anatum	1	<u>Shigella</u>	flexneri	3	1
"	lexington	1	"	boydii	11	1
"	tennessee	1	"	Boydii	13	2
"	isuge	1	"	alkalescens-dispar	01	1
"	newlands	1	"	alkalescens-dispar	04	<u>1</u>
"	Group B species	3				<u>6</u>
"	Group E species	3				<u>11</u>

### Vibrio Isolates

V. parahemolyticus K-19    1

### Enteropathogenic E. coli

0 86:B7	4
0 55:B5	1
0127:B8	2
0199:B14	1
0 25:B19:B23	4
0125:B15	10
0111:B4	6
0128:B12	<u>4</u>
	<u>32</u>

Neonatal diarrhea-related morbidity during August was zero among 371 deliveries. There were 11 cases of diarrhea, (0.3%). Demographically, the mothers were 41% from the Bangkok metropolitan area, 17% from suburbs, 18% from upcountry Thailand, and 23% of unknown address. No attempt was possible at this time to relate bacterial colonization with geographic home or to correlate symptomatic infant isolates with the maternal isolates. Of the 103 specimens, 50 were organisms of medical interest. Salmonella, Shigella and one Vibrio accounted for 36% of the significant isolates with the remaining 64% being types of enteropathogenic E. coli.

Forty eight percent (48%) of 103 mothers delivering had medical laboratory significant organisms upon admission to the delivery room. Further study of the infant and mother and family origins is suggested by this investigation as useful in determining the epidemiology of neonatal diarrhea as a significant medical problem in Thailand.

#### 4. Gibbon Release Program

OBJECTIVE: To release gibbons housed in the AFRIMS animal colony that were no longer utilized for research. Secondary objectives were to observe their ability to adapt to their natural habitat and their interaction with wild gibbons in the release area.

BACKGROUND: In January, 1976, it was decided that gibbons no longer utilized for research by the AFRIMS Medical Research Laboratory would be released in certain forested areas of Thailand.

The gibbons were selected for release based on breeding history, age, general health, compatibility and completion of research utilization. They were released either in family groups composed of the sire, dam and offspring; in pairs consisting of a compatible male and female with no offspring; or individually when an animal was found to be incompatible with all other available gibbons or had no suitable mate. Of the 31 gibbons selected for release 25 had spent six to eleven years in captivity and the remainder were colony born.

Several release areas situated throughout Thailand were studied and it was concluded that all gibbons would be released in the forested area of the Protein Expansion Project (PEP) at Sai Yok, Kanchanaburi. Several reasons were considered in the selection of this area, the most important of these being (1) it was a natural habitat of gibbons and contained wild groups of gibbons of the same species; (2) the native gibbon population was such that the area would accommodate the AFRIMS gibbons; (3) there was an abundance of fruit and water in the area, and (4) the area is protected by the Royal Thai Army and hunting is not allowed. Four separate release site were selected. Each site was separated by a distance of at least one kilometer from the others and was selected based on its proximity to humans, suitable terrain and location of native nonhuman primate family groups.

METHODS: Prior to release each gibbon was given a complete physical examination. A Certificate of Permission to Transport and Release was obtained from the Forestry Department for each animal determined to be in good health and suitable for release. In those cases that an animal was determined unsuitable for release, i.e. parasitic infestation, the entire family group was retained until the condition was corrected.

On the day of release each gibbon was tranquilized at the AFRIMS animal colony and transported to Sai Yok by vehicle. Upon arrival at Sai Yok each animal was anesthetized with Serylan (R), given a final visual examination and carried by hand to the pre-selected release site. At the release site the animals were observed until they were conscious and capable of normal movement.

A Thai employee of the PEP, was retained to monitor the activity of the gibbon following their release. Also periodic visits to each release site was made by AFRIMS personnel. On these visits the adaptation of the AFRIMS gibbon to its new environment was noted.

Table 1. Gibbons Released at Sai Yok-February 1976 to June 1977

<u>FAMILY GROUP</u>					
<u>Family#</u>	<u>Sire</u>	<u>Dam</u>	<u>Offspring</u>	<u>Date Released</u>	<u>Area*</u>
1	B82	Pc1	Pc33	9 July 1976	1
2	B8	B6	Pc20	11 May 1977	4
3	B64	B11	Pc32	25 May 1977	1
4	B163	B59	Pc35	8 June 1977	4
5	S18	V175	Pc36	15 June 1977	2

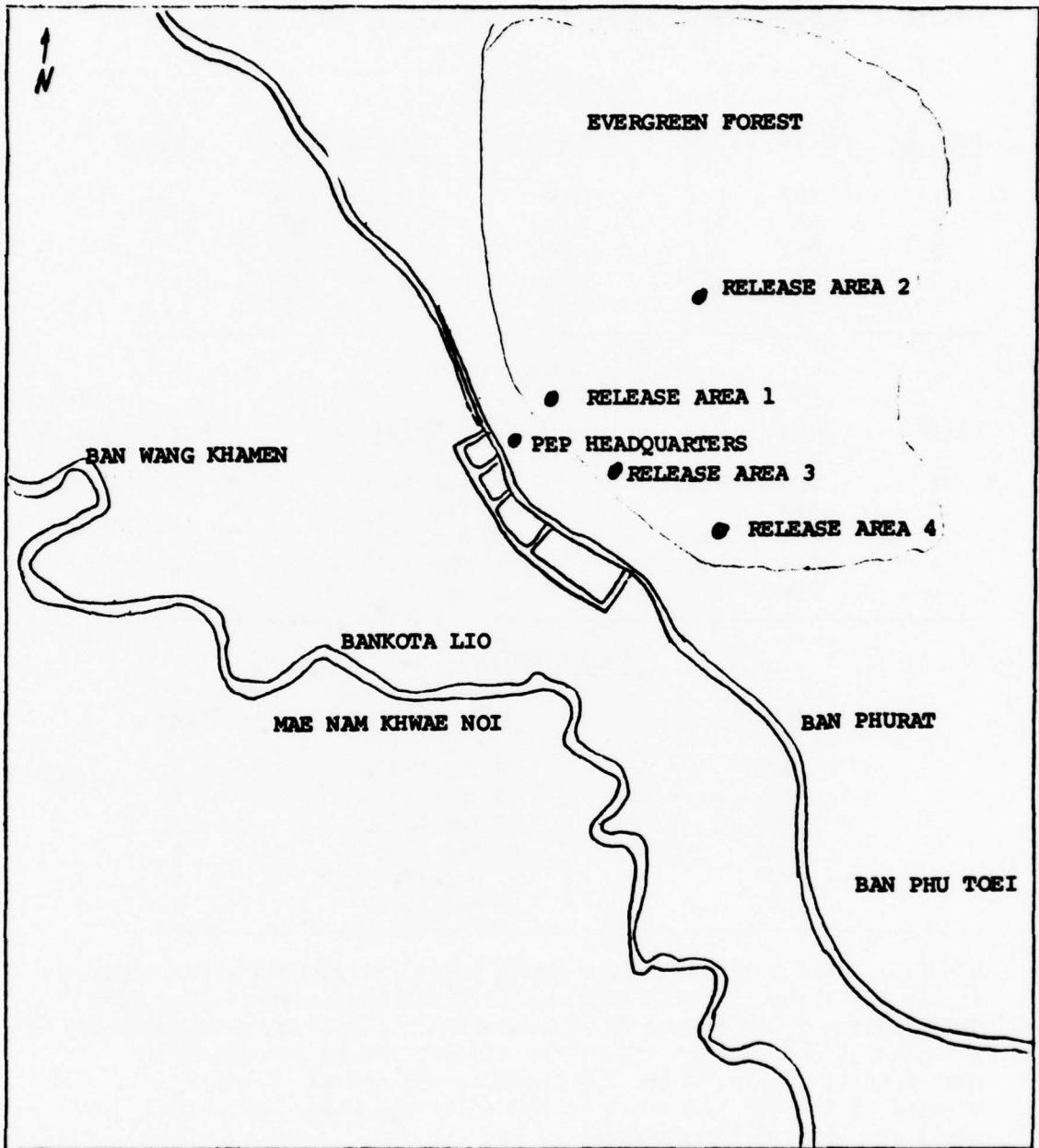
<u>PAIRS</u>					
<u>Pair#</u>	<u>Sire</u>	<u>Dam</u>	<u>Date Released</u>	<u>Area</u>	
1	VM61	B143	18 Feb 1976	1	
2	B83	P2	18 Feb 1976	1	
3	B61	B66S	9 June 1976	1	
4	B56	S81	21 Mar 1977	4	
5	P16	B7	20 Apr 1977	1	

<u>INDIVIDUALS</u>			
<u>Gibbon#</u>	<u>Sex</u>	<u>Date Released</u>	<u>Area</u>
S83	M	9 June 1976	3
P5	M	9 June 1976	3
B88	F	17 Aug 1976	1
B89	F	17 Aug 1976	4
B90	F	17 Aug 1976	4
S82	F	17 Aug 1976	1

RESULTS: During the seventeen month period 31 gibbons were released (table 1), three of which returned to local houses where they were captured and taken to the PEP headquarters. These were subsequently released at more remote sites; two did not return but the other continued to return to the PEP headquarters and was finally donated by the PEP personnel to the local Wat where he resided until the time of his death.

Only three of the gibbons are known to have died. One adult was found dead in the forest, one infant died of transportation stress and exposure within twelve hours after release and the animal donated to the Wat died of pneumonia.



Three gibbons were later observed in the company of wild gibbon families and two others were observed with bite wounds resulting from fights with wild gibbons.

At the present time there are seven gibbons in the AFRIMS animal colony still being utilized in research projects.

##### 5. Chemotherapy of Gnathostomiasis

OBJECTIVE: To continue to search for chemicals with chemotherapeutic activity against advanced third-stage larvae of Gnathostoma spinigerum.

BACKGROUND: These studies are a continuation of the work reported in previous years. Many antihelminthic drugs have been evaluated for possible chemotherapeutic activity against experimental G. spinigerum infection of white mice with advanced third-stage larvae or migrating stages of the worm. All drugs tested have been ineffective so far.

METHODS: Mice of the ICR strain were infected by oral administration each with five advanced third-stage larvae of G. spinigerum. After the infection was allowed to progress for some days, the test drug was administered parenterally in a predetermined regimen. Infected control mice were given the infection but no drug. After completion of the treatment regimen, the mice were sacrificed and necropsied. Parasites were counted in the liver, in other visceral organs, and in body muscles, and the results recorded.

Similarly, adult domestic cats, after being proved negative with natural Gnathostoma infection by monthly examination of stool for the Gnathostoma eggs by Formalin Ether Sedimentation technique (Ritchie) were infected each with 50-60 G. spinigerum advanced third-stage larvae (obtained from the experimentally infected mice) through skin penetration and oral infection. After the infection was allowed to proceed for 45-50 days, the test drug was administered parenterally according to the predetermined regimen. Infected control cats were given the infection but no drug.

The drug tested during this reporting period was Aencylol disophenol (2, 6-diiodo-4-nitrophenol) parenteral 4.5% (American Cyanamid Company, Princeton, N.J.)

RESULTS: The drug screening test on white mice infected with G. spinigerum advanced third-stage larvae gave the following results:

1. The drug was administered subcutaneously, five doses to each mouse, one dose per week, with the first dose of 0.1 ml/lb. body weight followed by 0.01 ml/lb. for 4 successive doses to Group A of 25 treated mice. Group B of 20 mice also received five doses each; the first and second doses had 0.1 ml/lb. body weight each, but other three successive doses had 0.01 ml/lb. body weight. These regimens were found to be ineffective in significantly reducing the numbers of the larvae in treated mice comparing with the control (Table 1).

2. The drug was administered subcutaneously, eight doses to each mouse, one dose/week, with each of the first three doses of 0.1 ml/lb. body weight and followed by each of five successive doses of 0.02 ml/lb. body weight to 19 white mice (Group A). A similar regimen was administered to another group of 20 white mice (Group B) after a shorter duration of the infection with the larvae than those of Group A mice. The numbers of larvae in treated mice of Group A were reduced compared with the control, but treated mice of Group B showed no significant reduction of the larvae compared with the control (Table 2).

Multiple subcutaneous administrations of Aencylol disophenol at the dosage of 0.1 ml/lb. body weight for 5-6 doses at weekly intervals has been giving good therapeutic effects on some cats and dogs experimentally infected with migrating stages of *G. spinigerum*, but some treated animals have shown signs of drug toxicity (3,4). The study initiated during this reporting period was to determine the multiple minimum effective doses of the drug for treating the migrating stages of the worm in cats without causing any toxicity.

With another treatment regimen, each of eight negative cats was infected by skin penetration and oral feeding with 50-60 *G. spinigerum* advanced third-stage larvae; two of these cats were designated as controls. The six infected cats were treated in groups of two by multiple subcutaneous infections of Aencylol at 10-day intervals for 12 doses with the dosages of 0.015 ml, 0.03 ml, and 0.02 ml per lb. body weight.

The results showed no worms in the organs of two cats treated with 0.05 ml parenteral Aencylol on necropsy 20 and 23 days after the last dose. Of two cats treated with the 0.02 ml dose, one had 10 living worms and 1 dead larva, and the other had 6 living larvae in the organs on necropsy 19 and 24 days after the last dose. The two control cats each had infection with 8 worms in the tissue. Thus 12 doses of 0.05 ml/lb body weight of

Table 1. Treatment of Gnathostoma spinigerum infected mice with Aencylol disophenol (2, 6-diiodo-4-nitrophenol) subcutaneous injection.

Drug dose per week		No. of mice treated	Mice positive with larvae	Third-stage larvae found no. (X)	Duration of infection of white mice before treatment (days)	Time of necropsy after treatment (days)	Remarks
0.1ml/lb. body weight	0.01ml/lb body weight						
<u>Group A</u>							
1	4	25	25	48(38.4)	16	11-13	
Control		10	10	20(40.0)	18	18	
<u>Group B</u>							
2	3	20	20	42(42.0)	61	1-13	
Control		10	10	41(42.0)	61	0-15	

Table 2. Treatment of *Gnathostoma spinigerum* infected mice with Aencylol disophenol (2, 6-diiodo-4-nitrophenol) subcutaneous injection.

Drug dose per week		No. of mice treated	Mice positive with larvae	Third-stage larvae found no. (%)	Duration of infection of white mice before treatment (days)	Time of necropsy after treatment (days)	Remarks
0.1ml/lb body weight	0.02ml/lb body weight						
<u>Group A</u>							
	3	5	19	18	28(29.4)	48-150	11-35
<u>Control</u>			15	15	31(41.3)	164-170	0-36
<u>Group B</u>							
	3	5	20	20	42(42.0)	15-58	12-27
<u>Control</u>							
			15	15	35(46.6)	7-15	0-27

Table 3 Chemotherapy of *Gnathostoma spinigerum* larval and immature stages infection in 6 adult cats each treated with 12 subcutaneous doses of Aencylol disophanol (2, 6-dido-4-nitrophenol) on the basis of 0.02 to 0.05 ml. per pound body weight at 10-day intervals.

Cat no.	Nos third-stage larvae given to cats (infection method)	Age of the worm in days in cats before treatment	Dose of Aencylol in ml. per lb body weight	Autopsy findings after completion of treatment		Days of sacrifice after last dose	Days of sacrifice after infection	Remarks
				Nos/stage of living worms	Organ found infected			
149	50(46 skin, 4 feeding)	45	0.05	0	0	20	176	-
150	50(47 skin, 3 feeding)	45	0.05	0	0	23	179	-
151	60(50 skin, 10 feeding)	46	0.03	0	0	24	181	-
152	52(35 skin, 17 feeding)	48	0.03	1 larva	Muscles of hind leg	22	181	-
153	55(35 skin, 20 feeding)	48	0.02	9 larvae	Muscles of abdominal wall, front and hind legs, chest	19	177	1 dead larva in muscles of chest
154	56(45 skin, 11 feeding)	50	0.02	1 immature 6 larvae	Muscles of chest, back and hind legs	24	184	
155	50(40 skin, 10 feeding) (Control)	-	-	4 larvae	Muscles of front & hind legs, chest, liver	-	191	
156	50(40 skin, 10 feeding) (Control)	-	-	4 immature (3♂, 1♀)	Muscles of chest, back & hind legs	-	-	
				4 larvae	Muscles of front & hind legs, abdominal wall	-	190	
				4 immature (3♂, 1♀)	Muscles of chest, back & diaphragm, stomach wall			

parenteral Aencylol given to cats at 10 day intervals is very effective chemotherapy in eliminating all migrating stages of Gnathostoma spinigerum in cats.

However, of the two cats treated with 12 doses of 0.03 ml/lb body weight, one showed no worms and the other had only one living larva in muscles of the leg (Table 3). It now remains to determine further that 0.04 ml/lb body weight of the chemical given in total of 12 doses to each cat would be the minimum effective chemotherapeutic treatment for G. spinigerum migrating stages infection in cats without causing toxicity.

All six cats treated with parenteral Aencylol in this experiment showed no signs of toxicity due to the drug on necropsy.

#### 6. Distribution of Rickettsia tsutsugamushi Strains in Thailand

OBJECTIVE: To determine the geographical distribution of various Rickettsia tsutsugamushi strains occurring in natural populations of vector chiggers in Thailand.

BACKGROUND: The vectors of scrub typhus feed on mammals only once during their life cycle. Transovarian transmission must therefore occur for the larval mite to act as a vector of the rickettsial organism. Consequently, examination of unfed larvae can establish whether wild-caught mites are infected with the disease organism. Formerly the detection of Rickettsia tsutsugamushi in different chigger vectors depended primarily on serological and clinical findings in laboratory animals. Animal isolation procedures are long and laborious, sometimes taking two to three months before the final results are determined. Now a technique using direct immunofluorescence has been developed to detect the rickettsia in naturally infected mites (5). Using this technique nine different strains of Rickettsia tsutsugamushi can be screened against, using the internal contents of each unengorged chigger.

METHODS: Unengorged chiggers were collected in Chiangmai, Korat, and Ubon Provinces from various types of habitat such as grass-land, scrub and forest. Formica black plates measuring 5" x 5" were used to find chiggers that were resting on leaves or grass. Larval mites seen moving across the plates were picked-up with a moisten applicator stick and placed into screwcap vials containing water. Collections of live chiggers were then sent to USAMRU in Malaysia for testing by direct immunofluorescence. Identification of the chiggers was also done at this time. The nine strains of

Rickettsia tsutsugamushi used in the preparation of conjugates in this study were as follows: Karp, Gilliam, Kato, TA678, TA586, TA686, TA716, TA763, and TH1817. The first three strains are prototype scrub typhus strains and the other six are strains originally isolated from Thailand.

Chiggers were placed individually in five lambda of 0.5% normal yolk sac suspension. The exoskeleton of the chigger was punctured dorsal-posteriorly and the internal contents squeezed out. The contents were spotted in ten predetermined areas on a microscope slide with four mites being tested each time. Slides were fixed with carbon tetrachloride and dried at room temperature. The exoskeleton of the mite was mounted in a drop of Hoyer's mounting media for species identification.

Fixed slides stored at -20°C were warmed to room temperature before testing by direct immunofluorescence. Each group of spots was ringed with nail polish and the strain-specific conjugates were added to the spots. Slides were read within 24 hours.

RESULTS: Over 2,400 chiggers were collected off black plates at 24 different locations in the three provinces. Most of the mites collected were Leptotrombicula (L.) deliense. Not all chiggers were analysed in USAMRU-Kuala Lumpur due to mortality and loss of chiggers from the collecting vials. The results of the direct immunofluorescence test to detect the presence of Rickettsia tsutsugamushi in the different species of mite are not yet complete and will be reported later.

#### 7. Pathogens of Medically Important Mosquitoes of Thailand

OBJECTIVE: To determine the kinds of mosquito pathogens occurring in medically important mosquitoes in Thailand and to elucidate the biology of selected pathogens sufficiently to assess their potential as biological mosquito control agents.

BACKGROUND: Results of surveys to determine the kinds and distributions of mosquito pathogens infecting Aedes aegypti and Culex pipiens quinquefasciatus in Thailand were previously reported (6, 7). Among the 16 host-pathogen systems found in these species, 13 were considered worthy of evaluation as potential biological control agents; i.e., the pathogens represented taxa containing organisms known to be pathogenic to mosquitoes. Unfortunately, some of these systems were rare or were found far from the laboratory. Evaluation of biological control potential was

initiated with six accessible systems. These represented what appeared to be four species of pathogens, two of which occurred in both A. aegypti and C. pipiens quinquefasciatus. Microsporidian #1, probably Stempellia milleri, was found in C. pipiens quinquefasciatus. Microsporidian #2, possibly also of Genus Stempellia, since pansporoblasts with both four and eight sporoblasts occur in its developmental cycle, occurred in A. aegypti. An helicosporidian, near Helicosporidium parasiticum, occurred in both A. aegypti and C. pipiens quinquefasciatus throughout Thailand. A minute, and as yet unculturable and unidentified, gram negative, motile, vibrioform bacterium also occurred in both host pseclies and has been observed to be the probable cause of massive spizooootics in larval C. pipiens quinquefasciatus.

The general approach to evaluating biological control potential in the laboratory consisted of, first, establishing the pathogens in the laboratory and, then, conducting experiments with them to determine the degree to which they possessed characteristics desired in biological control agents for mosquitoes. Since no pathogen has yet been developed for large scale use against mosquitoes, desired "standards of performance" were hypothetical. A biological agent to be used against mosquitoes should possess at least the following characteristics: (1) it should kill mosquitoes or otherwise interfere with their ability to transmit diseases, (2) its effects on non-target organisms should be ecologically acceptable, (3) the cost of its production and use should be acceptable, (4) it should have a shelf-life of acceptable duration, and (5) it should not be neutralized by the environment into which it is dispersed until it has infected the desired proportion of the target population.

METHODS: Methods commonly used will be described here. Those specific to certain experiments will be described where appropriate. Mosquito larvae infected with the pathogens of immediate concern displayed gross signs of pathology which permitted the identification and collection of heavily infected larvae in the field. These were transported to the laboratory and used as a source of inoculum for the establishment of pathogens in laboratory reared mosquitoes. Once this was accomplished, laboratory produced pathogens were used as inocula for experiments.

The stock mosquito colony was maintained in a separate insectary into which pathogens were never taken, using equipment kept only in that insectary. Pathogen suspensions for use as inoculum were prepared by triturating infected hosts in a Ten-Broeck type tissue grinder until the head capsules were destroyed. The tritulant was filtered through organdy cloth to remove large

debris and washed by centrifugation. Protozoan pathogens (helicosporida and microsporida) were quantified using a hemocytometer. Bacterial inoculum was quantified crudely in units of larval equivalents, one larval equivalent being the bacteria from one patently infected larva. Per os exposures of uninfected larvae were made in 85 mm plastic petri dishes that were washed and ethanol sterilized between usages. Routinely, 200 mosquito larvae were placed in these with 20 ml of pathogen suspension. A small quantity of food was provided during exposure. Except in experiments where duration of exposure was a variable, exposures were of 24 hr. duration. Following exposure, the larvae were poured onto an organdy cloth screen, gently rinsed with tap water, and then placed in rearing pans. The contents of one exposure container, 200 larvae, were placed in one pan. Post exposure rearing was in enamel or aluminum pans that were sterilized by autoclaving after use. Larvae were fed laboratory animal chow ground finely enough to pass through a 60 mesh screen.

Specimens for examination were usually collected from among the exposed larvae when pupae first appeared. It was advantageous to allow infections to develop as long as possible to facilitate their detection. However, many mosquito pathogens kill their hosts in the late fourth stage or during pupation, and this mortality makes it difficult to collect a random sample representing the exposed population.

Infections were detected by smearing mosquitoes onto microscope slides with the tips of wooden applicator sticks, five discrete smears per slide. The smears were air-dried, fixed for one minute with absolute methanol, and stained with Giemsa stain in 0.01 M pH 7.6 Tris buffer. Coverslips were applied, and the smears were examined at 500X or 1250X magnification. No less than 50 individuals from any experimental group were examined, except where indicated. Dose-response data at several dosages were commonly collected. These data were plotted on similog graph paper, regression lines were eye-fitted, and IC<sub>50</sub>'s were estimated from these. The stock colony was routinely sampled to assure that it was free from pathogens.

#### ESTABLISHMENT OF PATHOGENS IN THE LABORATORY

##### Microsporidan #1 in *Culex pipiens quinquefasciatus*

Methods: Field collected *Culex pipiens quinquefasciatus* had 1.7 x 10<sup>6</sup> spores per infected larva. Twenty-four hour old laboratory produced *C. pipiens quinquefasciatus* larvae were exposed to 3.4 x

$10^6$  spores per ml.

Results: Seventy-seven percent transmission occurred among 100 specimens examined. Other transmission attempts using fewer spores or older larvae were unsuccessful. This pathogen was not routinely maintained in the laboratory, but it appears very likely that it could be.

Microsporidan #2 in *Aedes aegypti*

Methods: *Aedes aegypti* larvae from several localities had from  $2.5 \times 10^5$  to  $3.8 \times 10^5$  (mean =  $3.04 \times 10^5$ ) spores per infected larva. Twenty four hour old laboratory reared *A. aegypti* larvae were exposed.

Results: Dosages of  $4 \times 10^3$ ,  $8 \times 10^3$ ,  $2 \times 10^4$ ,  $4 \times 10^4$  and  $2 \times 10^5$  spores per ml produced 7, 16, 30, 84 and 100 percent infection, respectively, with an IC<sub>50</sub> of between  $3 \times 10^4$  and  $4 \times 10^4$  spores per ml. This pathogen was routinely maintained in the laboratory with ease.

Microsporidan #2 in *Culex pipiens quinquefasciatus*

Methods: Forty-eight hour old *Culex pipiens quinquefasciatus* larvae were exposed to  $1 \times 10^6$  spores per ml.

Results: No transmission was detected.

*Helicosporidium* sp. in *Culex pipiens quinquefasciatus*

Methods: Field collected *Culex pipiens quinquefasciatus* larvae infected with this pathogen averaged  $2.25 \times 10^6$  spores per larva. Early second stage larvae were exposed.

Results: Dosage of  $1 \times 10^4$ ,  $2 \times 10^4$ ,  $5 \times 10^4$ ,  $1 \times 10^5$  and  $2.5 \times 10^5$  spores per ml produced 8, 32, 76, 84 and 100 percent infection, respectively, with an IC<sub>50</sub> of about  $1.35 \times 10^5$  spores per ml.

*Helicosporidium* sp. in *Aedes aegypti*

Methods: *Aedes aegypti* larvae infected with this pathogen display much less conspicuous signs of infection than do infected *Culex pipiens quinquefasciatus*. Therefore, field collected *Culex* were initially used as a source of inoculum to establish this pathogen in *A. aegypti* by exposing 48 hr. old larvae to various dosages.

Results: Difficulty was experienced establishing this pathogen in A. aegypti. Dosages routinely used to infect Culex caused massive mortality. Eventually, by exposing A. aegypti to much lower dosages, a highly efficient system for the production of this pathogen was developed. Exposure of 48 hr. old larvae to dosages of  $5 \times 10^2$ ,  $1 \times 10^3$ ,  $5 \times 10^3$ , and  $1 \times 10^4$  spores per ml produced 8, 24, 90 and 100 percent infection, respectively, with an  $IC_{50}$  of about  $1.9 \times 10^3$ .

#### Helicosporidium sp. in Anopheles balabacensis

Methods: Both second and third stage Anopheles balabacensis were exposed to Helicosporidium spores produced in the laboratory in Aedes aegypti.

Results: Exposure of second stage An. balabacensis larvae to dosages of  $1 \times 10^3$ ,  $5 \times 10^3$ ,  $1 \times 10^4$  and  $5 \times 10^4$  spores per ml produced 60, 96, 100 and 100 percent infection, respectively, with an  $IC_{50}$  of less than  $1 \times 10^3$  spores per ml. Exposure of third stage larvae to dosages  $1 \times 10^3$ ,  $5 \times 10^3$ ,  $1 \times 10^4$ ,  $5 \times 10^4$ ,  $1 \times 10^5$ ,  $5 \times 10^5$  and  $1 \times 10^6$  spores per ml produced 8, 16, 20, 56, 88, 100 and 100 percent infection, respectively, with  $IC_{50}$  of about  $2.5 \times 10^4$ . The sharp increase in the  $IC_{50}$ 's with age of larvae at exposure was commonly encountered.

#### Helicosporidium sp. in Anopheles maculatus

Methods: Anopheles maculatus from Malaysia, where the species is an important vector of human malaria, were exposed as second stage larvae to Helicosporidium spores produced in Aedes aegypti.

Results: Exposure of larvae to dosages of  $7.5 \times 10^3$ ,  $11.25 \times 10^3$ , and  $1.5 \times 10^4$  spores per ml produced 10, 22 and 48 percent infection, respectively, with a probable  $IC_{50}$  of about  $1.7 \times 10^4$  spores per ml.

#### Unidentified Bacterium in Culex pipiens quinquefasciatus

Methods: Late second stage Culex pipiens quinquefasciatus larvae were exposed to 0.75 larval equivalents per ml of this agent alone and along with  $5 \times 10^5$  Helicosporidium spores per ml.

Results: No transmission of the bacterium occurred to larvae exposed to it alone. In the groups exposed to both the bacterium and to the protozoan, mortality was very high in early larval stages. Of those surviving to the fourth stage, 12 percent were infected with Helicosporidium alone, 8 percent were infected with the

bacterium alone and 2 percent were infected with both pathogens. Because of the high mortality occurring soon after exposure, these results probably are deceptive. In experiments described below, larval mortality occurring soon after exposure appeared to be due to septicemia, and a possible mechanism is discussed.

#### EXPERIMENTS PERTINENT TO EVALUATION OF BIOLOGICAL CONTROL POTENTIAL

##### Effects of Age of *Aedes aegypti* Larvae at Exposure on Percent Transmission of Microsporidan #2

Methods: *A. aegypti* larvae were exposed to  $5 \times 10^4$  spores per ml of Microsporidan #2 during the second, third, fourth and fifth days of larval life. These ages corresponded to the second, early third, early fourth, and late fourth larval stages, respectively.

An additional group was exposed to  $1 \times 10^5$  spores per ml during the third day of life, when the larvae were in the early third stage.

Results: Transmission rates of 100, 2, 0 and 0 percent respectively, were achieved with the first four groups, indicating a precipitous decrease in susceptibility with increasing age. One-hundred percent transmission was achieved in the group exposed to  $1 \times 10^5$  spores per ml, indicating that an increase in dosage could compensate for the decrease in susceptibility, at least in larvae of the age tested.

##### Vertical transmission of Microsporidan #2 in *Aedes aegypti*

Methods: *Aedes aegypti* larvae, 24 hrs. old, were exposed to  $7.5 \times 10^4$  spores per ml. When pupation began, smears were made of a random sample of fourth stage larvae, and percentage transmission was determined. Pupae were collected and surviving adults were given a blood meal and allowed to oviposit. Samples of the ova were hatched after one week and 10 weeks storage at room temperature. Resulting larvae were reared to advanced fourth stage, and the percent infected was determined. Adult survivors of the larvae hatched after one week storage were allowed to oviposit, and these progeny and their progeny were checked for infection with the microsporidam.

Results: Infection occurred in 95 percent of the exposed larvae. The progeny of the exposed generation hatched from ova stored for only one week were 45 percent infected. After storage for

an additional nine weeks, the percent infection in progeny was reduced to 4 percent, suggesting that ova carrying the infection died more rapidly than those which did not. No transmission was detected in generations after the immediate progeny of the exposed generation.

Effects of Age of *Aedes aegypti* Larvae at Exposure on Percent Transmission of *Helicosporidium* sp.

Methods: Groups of *Aedes aegypti* larvae of increasing ages were exposed to the dosages of *Helicosporidium* spores indicated in Table 1. When pupation began, 100 larvae from each experimental group were examined to determine percent infection. IC<sub>50</sub>'s were estimated from dose-response data. Infections in the remaining larvae were allowed to develop until the larvae died or pupated, at which time they were held at 4°C. When all larvae had died or pupated, a random sample of 100 from each age group were triturated, and the number of spores produced per infected individual was determined.

Results: (See Table 1) IC<sub>50</sub> was lowest for larvae exposed for 24 hrs. beginning at 48 hrs. of age. This was unexpected and will be discussed briefly below. The number of spores produced per infected larva was inversely proportional to age of larvae at exposure, reflecting the greater time available for development of infections in larvae that were younger when exposed. It is also possible that younger larvae were less resistant to penetration of the infectious agent, and, therefore, received a heavier initial dose.

Mortality was not quantified in this experiment, but it was noticed to be bimodal. One peak occurred soon after exposure and another in the late fourth larval stage. Mortality in the first peak seemed to be heaviest in younger larvae exposed to heavier dosages. Developing *Helicosporidium* was seldom found in these larvae. All were septicemic. Possibly, bacteria penetrated the gut wall along with the sporozoites of *Helicosporidium* and multiplied in the hemolymph rapidly enough to kill the larvae before the protozoan infection developed. The unexpectedly high IC<sub>50</sub>'s in young larvae might have resulted from high mortality occurring soon after exposure among younger infected larvae. Differential mortality in these groups would distort dose-response data in such a way as to lower IC<sub>50</sub>'s derived from them.

Forty-eight hour old larvae were considered ideal for dose-response experimentation. They were strong enough to survive

exposure, yet, enough time remained in the larval stage for most infections to be well developed before the second mortality peak began decimating the exposed populations.

Effects of Duration of Exposure of *Aedes aegypti* Larvae on Percent Transmission of *Helicosporidium* sp.

Methods: Groups of *Aedes aegypti* larvae, 48 hrs. old, were given exposures of increasing duration to a range of dosages of *Helicosporidium* spores. When the exposures at each duration were completed, the larvae were placed on organdy cloth screens and rinsed gently with tap water, before returning them to a clean exposure container until the exposure of maximum duration was completed. Following exposure, rearing procedures described above were followed, and random samples to determine percent transmission were taken of the exposed larvae when pupation began. IC<sub>50</sub>'s were estimated from dose-response data.

Results: As indicated in Table 2, an inverse relationship existed between duration of exposure and percent transmission. Brief exposures produced 100 percent transmission only if dosage was high. Twenty-four hour exposures were adopted as routine, because exposures of that duration required only about one-half as much inoculum to effect 100 percent transmission as required by one hour exposures. Larval mortality, though not quantified, seemed to increase with both duration of exposure and with dosage. If minimum dosages required to produce 100 percent infection in larvae of the age being used were administered, mortality was not a serious problem until infections were well developed.

Relationship Between Intensity of Exposure of *Aedes aegypti* to *Helicosporidium* Spores and Percent Infection, Mortality and Time of Occurrence of Mortality

Methods: Three groups of 2400, 48 hr old *Aedes aegypti* larvae were exposed for 24 hrs. to three different dosages of *Helicosporidium* spores,  $1 \times 10^4$ ,  $3 \times 10^4$  and  $9 \times 10^4$ . One dose was intended to give less than 100 percent infection, one dose was intended to give 100 percent infection without overwhelming the host; and one dose was intended to grossly overwhelm the host. Daily mortality data were recorded, until the mosquitoes were 15 days old, to determine the relationship between dose and mortality and time of occurrence of mortality. Beginning with mosquitoes that died between 24 and 48 hrs. after the initiation of exposure, the percentage of infection detectable in dead mosquitoes was determined at 24 hr. intervals until the

mosquitoes were 11 days old. Beginning 48 hrs. after the initiation of exposure, daily samples of live larvae, larvae and pupae, pupae, pupae and adults, or adults (whichever forms were prevalent at the time of sampling) were examined to determine the percentage of infection in the proportion of the population still alive, until the mosquitoes were 11 days old. Controls were exposed to a tritulant of uninfected larvae and reared in parallel with the experimental groups.

Results: Only 4 percent mortality and no infection occurred in the control group during the two weeks of the experiment. This mortality was distributed throughout the development cycle of the mosquitoes, with 38.6 percent of total mortality occurring among larvae, 38.6 percent among pupae, and 22.8 percent among adults. The control data indicated that conditions of exposure and rearing were not unduly harmful to the mosquitoes.

Mortality among the group exposed to  $1 \times 10^4$  spores per ml was 62.3 percent. Of that mortality, 27.4 percent occurred among larvae, 57.4 percent among pupae, and 15.2 percent among adults. Mortality among the group exposed to  $3 \times 10^4$  spores per ml was 92.8 percent. Of that mortality, 36.7 percent occurred among larvae, 54.5 percent among pupae, and 8.8 percent among adults. Mortality among mosquitoes exposed to  $9 \times 10^4$  spores per ml was 98.0 percent. Of that mortality, 79.5 percent occurred among larvae, 18.0 percent among pupae, and 2.4 percent among adults. Daily occurrence of mortality is shown in Table 3, and the bimodality of mortality is evident. An increasing proportion of total mortality occurred soon after exposure as dosage increased. Also, the second peak of mortality occurred earlier as dosage increased, indicating that time of mortality was related to intensity of exposure. This was reflected in the distribution of mortality between larvae, pupae and adults in the three experimental groups.

Actual percentages of infection resulting from exposure to Helicosporidium were difficult to determine. Difficulties resulting from mortality soon after exposure were mentioned above. As illustrated in Table 4, percentage of infection that could be detected varied with time since exposure and with mortality that had occurred prior to collecting samples for examination. Among larvae exposed to relatively light doses, not only did less transmission occur, but infections were more difficult to detect soon after exposure. A smaller proportion of total infections were found to occur, detected 48 or 72 hrs. after initiation of exposure, in lightly exposed groups than in heavily exposed groups. Also deceptive were percents of infection taken

after the second peak of mortality. Only 60 percent infection was detected in adults surviving for nine days after initiation of the heaviest exposure. Samples taken as early as the fourth day after initiation of exposure in that group were 98 percent infected. Most of the exposed population (95.8 percent) died before the ninth day after initiation of infection, and the few uninfected individuals became a conspicuous proportion of the remaining population.

The most accurate single determination of percent transmission could be made from samples collected just before the occurrence of the second mortality peak. By that time, most infections became detectable by the methods used, and distortions of dose-response data caused by the second mortality peak were not yet a problem. In all three exposure groups, the second mortality peak began on day six after the initiation of exposure, so dose-response data from day five should be the most accurate. The peak in mortality rate occurred one day earlier for each progression in dosage.

#### Retention of Infectivity by *Helicosporidium* Spores Stored in Demineralized Water at 4°C

Methods: Spores were recovered from infected *Aedes aegypti* larvae and washed three times by centrifugation at 750 x g for 20 min at 4°C. The spores were divided into aliquots and stored in a refrigerator in demineralized water at 4°C in cotton-stoppered glass test tubes. Baseline dose-response data was acquired with a portion of the spores before storage. After one and two weeks of storage and biweekly thereafter until 16 weeks of storage, aliquots of the spores were evaluated for infectivity.

Results: As indicated in Table 5, considerable loss of infectivity occurred during the 16 week storage period, but storage at 4°C for short periods should be useful to investigators. Spore counts per unit volume remained essentially the same throughout the storage period, indicating no degeneration of physical integrity of the spores.

#### Retention of Infectivity by *Helicosporidium* Spores at Room Temperature in Buffer Solution and in Buffered Solution of Antibiotics

Methods: Spores were recovered from infected *Aedes aegypti* larvae and washed three times by centrifugation at 750 x g for 20 min at 4°C in phosphate buffered saline, pH 7.0. After the

final wash, aliquots of the spores were resuspended in the appropriate storage medium. Four storage media were tested: (1) 0.05 M phosphate buffer, pH 7.0, (2) 400 units of Potassium Penicillin G and 1.0 mg Streptomycin Sulfate per ml of the same buffer, (3) 1000 units of Polymyxin B Sulfate per ml of buffer, and (4) 1.5 mg Kanamycin Sulfate per ml of buffer. Baseline dose-response data were acquired with untreated spores. Suspensions of the treated spores were stored in the dark at 25°C, and infectivity was tested at intervals of 10, 17 and 24 days.

Results: No transmission was acquired with spores stored in Penicillin-Streptomycin or in Kanamycin. As indicated in Table 6, some infectivity was retained by spores stored in buffer and in buffered Polymyxin B for up to 17 days. No transmission occurred with spores stored for 24 days.

Retention of Infectivity by Helicosporidium Spores Stored by Lyophilization, Vacuum Drying and Freezing \*

Methods: Spores of Helicosporidium were preserved in intact, infected Aedes aegypti larvae by freezing in liquid nitrogen (-196°C) and were then stored in liquid nitrogen and in a REVCO freezer at -70°C. Spores in intact larvae were also preserved by lyophilization and by vacuum drying and stored at -70°C and at room temperature. Spores recovered from infected larvae by trituration and centrifugation were preserved by freezing in liquid nitrogen as suspensions in two cryo-protectants, one based on dimethyl sulfoxide (8) and one based on egg yolk and glycerine (9). These were stored in liquid nitrogen and at -70°C. Baseline dose-response data was acquired with fresh spores. At intervals of 4, 8, 12 and 16 weeks, infectivity of the spores was evaluated and dose-response data acquired. This data was used to eye-fit curves of the regression of dosage on percent transmission. IC<sub>50</sub>'s were estimated from the regression lines.

Results: Some transmission was acquired after four weeks with spores subjected to all preservation and storage methods. However, methods employing drying permitted very poor retention of infectivity. The IC<sub>50</sub>'s of spores preserved by lyophilization or vacuum drying increased by more than a factor of 100 (2 logs). Storage and preservation by all methods involving freezing without drying were highly effective, as shown in Table 7.

\*This project constituted the research for a Master of Science Degree for Miss Boongea Witethom, Department of Biology, Chulalongkorn University, Bangkok, Thailand.

Many of the IC<sub>50</sub>'s for stored material were lower than the IC<sub>50</sub>'s for fresh spores. All IC<sub>50</sub>'s for the 16th week of storage were less than those after only four weeks of storage. The temperatures in the insectaries in which the experimental larvae were reared was uncontrolled. The experiments were done during a season of generally decreasing ambient temperatures. All tests were initiated with 48 hr. old larvae, but, as the temperature dropped or rose from week to week during the 48 hr. pre-exposure incubation period, the larvae against which spore infectivity was tested were smaller or larger; i.e., their biological age was less or more. The sensitivity of percent transmission to age of larvae at exposure was shown above. It was hypothesized that temperature variation precipitated the aberrant results. These data illustrate the futility of attempting quantitative experimental pathology under uncontrolled conditions.

#### Preliminary Observations on the Infectivity of Helicosporidium Spores to the Golden Hamster

Method: Eleven adult male and one adult female golden hamsters were given 40 x 10<sup>6</sup> Helicosporidium spores in drinking water over a 12 hr. period. For 30 days they were observed for weight loss and behavior changes. After 30 days, they were weighed and necropsied. Observations for gross pathology were made and samples of organs were preserved in neutral buffered formalin for histological examination (not yet done).

Results: No weight loss or change in behavior was observed. The eleven males appeared normal when examined for gross pathology. Pyometra was observed in one uterine horn of the single female examined. Results of histological examinations will be reported when completed.

#### Infectivity of Helicosporidium sp. for Toxorhynchites splendens, a Mosquito Predator of Mosquitoes

Methods: One-hundred first stage and 100 third stage Toxorhynchites larvae were exposed for 24 hrs. to 1 x 10<sup>6</sup> Helicosporidium spores per ml. Following exposure, they were fed a liberal diet of uninfected Aedes aegypti larvae of appropriate size until they either died or pupated. Dead larvae and pupae were examined for presence of Helicosporidium as described above.

One-hundred third stage Toxorhynchites larvae were fed a diet of three known Helicosporidium infected fourth stage A. aegypti larvae for five successive days. Subsequent to exposure, the Toxorhynchites larvae were fed a diet of uninfected larvae until they either died or pupated. Dead larvae and pupae were smeared and examined for Helicosporidium infections as described above.

Table 1. Effects of age of Aedes aegypti at exposure to  
Helicosporidium sp. on percent transmission and spore  
production.

Age at Exposure	Dose (Spores/ml)	Percent Transmission	Spores Produced Per Infected Larva
(IC <sub>50</sub> = 2 x 10 <sup>4</sup> )	1 x 10 <sup>3</sup>	10	3.4 x 10 <sup>6</sup>
	5 x 10 <sup>3</sup>	18	3.0 x 10 <sup>6</sup>
	1 x 10 <sup>4</sup>	32	2.4 x 10 <sup>6</sup>
	2 x 10 <sup>4</sup>	42	1.5 x 10 <sup>6</sup>
	3 x 10 <sup>4</sup>	68	1.1 x 10 <sup>6</sup>
(IC <sub>50</sub> = 4 x 10 <sup>3</sup> )	1 x 10 <sup>3</sup>	12	3.6 x 10 <sup>6</sup>
	5 x 10 <sup>3</sup>	46	1.7 x 10 <sup>6</sup>
	1 x 10 <sup>4</sup>	92	1.3 x 10 <sup>6</sup>
	2 x 10 <sup>4</sup>	100	1.3 x 10 <sup>6</sup>
	5 x 10 <sup>4</sup>	100	1.5 x 10 <sup>6</sup>
(IC <sub>50</sub> = 1.9 x 10 <sup>3</sup> )	1 x 10 <sup>3</sup>	28	4.3 x 10 <sup>5</sup>
	5 x 10 <sup>3</sup>	70	6.0 x 10 <sup>5</sup>
	1 x 10 <sup>4</sup>	86	7.0 x 10 <sup>5</sup>
	2 x 10 <sup>4</sup>	98	7.5 x 10 <sup>5</sup>
	5 x 10 <sup>4</sup>	100	9.5 x 10 <sup>5</sup>
(IC <sub>50</sub> = 3 x 10 <sup>5</sup> )	5 x 10 <sup>3</sup>	8	1.3 x 10 <sup>5</sup>
	1 x 10 <sup>4</sup>	6	8.1 x 10 <sup>5</sup>
	3 x 10 <sup>4</sup>	18	5.3 x 10 <sup>4</sup>
	5 x 10 <sup>4</sup>	20	2 x 10 <sup>4</sup>
	7 x 10 <sup>4</sup>	42	1.4 x 10 <sup>5</sup>

Table 2. Percent transmission of Helicosporidium to Aedes aegypti larvae following exposures of increasing duration.

Dose (Spores/ml)	Duration of Exposure (Hours)					
	1	4	8	16	24	32
$5 \times 10^2$	4	4	6	6	8	10
$1 \times 10^3$	6	10	14	20	24	28
$5 \times 10^3$	46	72	80	88	90	95
$1 \times 10^4$	94	100	100	100	100	100
$5 \times 10^4$	100	100	100	100	100	100
IC <sub>50</sub> 's	$3.5 \times 10^3$	$2.6 \times 10^3$	$2.2 \times 10^3$	$2.0 \times 10^3$	$1.9 \times 10^3$	$1.8 \times 10^3$
						$1.45 \times 10^3$
						$1.4 \times 10^3$

Table 3. Percentage of total mortality and cumulative mortality that occurred on each day after initiation of exposure.

Day After Init. of Exp.	Dosage				% of Total Mortality	Cumulative Mortality	% of Total Mortality	Cumulative Mortality	% of Total Mortality	Cumulative Mortality
	1 x 10 <sup>4</sup>	3 x 10 <sup>4</sup>	9 x 10 <sup>4</sup>	9 x 10 <sup>4</sup>						
1	0	0	0.1	0.1	0	0	0.1	0.1	0.8	0.8
2	1.1	1.1	2.6	2.7	1.1	2.6	2.7	22.3	23.1	23.1
3	0.6	1.7	0.7	3.4	1.7	0.7	3.4	6.5	29.6	29.6
4	0.4	2.1	0.4	3.8	2.1	0.4	3.8	2.6	32.2	32.2
5	0.2	2.3	0.6	4.4	2.3	0.6	4.4	1.9	34.1	34.1
6	6.6	8.9	16.4	20.8	8.9	16.4	20.8	7.4	41.5	41.5
7	17.9	26.8	20.3	41.1	26.8	20.3	41.1	25.2	66.7	66.7
8	24.2	51.0	34.8	75.9	51.0	34.8	75.9	19.8	86.5	86.5
9	32.0	83.0	21.3	97.2	83.0	21.3	97.2	9.4	95.5	95.5
10	8.3	91.3	2.0	99.2	91.3	2.0	99.2	2.4	98.3	98.3
11	5.8	97.1	0.5	99.7	97.1	0.5	99.7	1.0	99.3	99.3
12	2.4	99.5	0.3	100.0	99.5	0.3	100.0	0.4	99.7	99.7
13	0.5	100.0	0.1	100.1	100.0	0.1	100.1	0.2	99.9	99.9

Table 4. Percentages of infection with Helicosporidium detected in Aedes aegypti larvae.

Day After Initiation of Exposure	Form (L)=Live (D)=Dead	Dosage (Spores/ml)					
		#Exam.	1 x 10 <sup>4</sup>	#Exam.	3 x 10 <sup>4</sup>	#Exam.	9 x 10 <sup>4</sup>
2	(L) Larvae	50	38	50	86	50	94
	(D) Larvae	7	28	12	41	50	42
3	(L) Larvae	50	52	50	90	50	92
	(D) Larvae	5	40	7	71	50	44
4	(L) Larvae	50	58	50	84	50	98
	(D) Larvae	2	50	10	100	35	91
5	(L) Larvae	50	84	50	100	50	100
	(L) Pupae	50	44	50	98	50	94
6	(D) Larvae	47	48	50	92	50	100
	(D) Pupae	45	54	50	98	38	100
	(L) Pupae	50	70	50	94	50	100
	(D) Larvae	50	68	50	100	50	100
	(D) Pupae	50	74	50	98	50	100

Table 4. continued

Day After Initiation of Exposure	Form (L)=Live (D)=Dead	Dosage (Spores/ml)					
		$1 \times 10^4$		$3 \times 10^4$		$9 \times 10^4$	
#Exam.	%Inf.	#Exam.	%Inf.	#Exam.	%Inf.	#Exam.	%Inf.
7	(D) Pupae	50	96	50	100	50	100
	(D) Adults	50	80	50	98	25	96
8	(D) Adults	50	90	50	98	23	96
9	(L) Adults	50	18	50	66	40	60

Table 5. Percentages of infection acquired by exposing 48 hr old *Aedes aegypti* larvae to suspensions of *Helicosporidium* spores stored in demineralized water.

Dose (Spores/ml)	Duration of Storage (weeks)						
	Fresh Spores	1	2	4	6	8	10
$5 \times 10^3$	86	90	90	88			
$1 \times 10^4$	100	100	98	100	68	80	
$2 \times 10^4$	100	100	100	100			
$3 \times 10^4$					100	100	
$5 \times 10^4$	100	100	100	100			
$7 \times 10^4$					100	100	
						100	100

Table 6. Percentages of infection acquired with *Helicosporidium*  
spores stored at room temperature in phosphate buffer  
and in phosphate buffered Polymyxin B.

Dose (Spores/ml)	Baseline % Inf.	Storage Medium			
		0.05M. Phosphate Buffer		Buffered Polymyxin B	
		10 days	17 days	10 days	17 days
$5 \times 10^3$	50				
$1 \times 10^4$	72	4	0	26	0
$2.5 \times 10^4$	100				
$3 \times 10^4$		6	0	83	0
$5 \times 10^4$	100	10	0	68	4
$1 \times 10^5$		24	2	66	14
$3 \times 10^5$		28	6	57	26

Table 7. IC's of Helicosporidium Spores Stored for up to 16 Weeks by Freezing.  
50

Material	<u>Preservation</u> Storage	Weeks of Storage			
		4	8	12	16
Spores in intact larvae	Liquid Nitrogen	3.5 x 10 <sup>3</sup>	1.0 x 10 <sup>4</sup>	10 <sup>4</sup>	2.1 x 10 <sup>3</sup>
	Liquid Nitrogen				
Spores in intact larvae	Liquid Nitrogen	3 x 10 <sup>3</sup>	4.5 x 10 <sup>3</sup>	2.5 x 10 <sup>3</sup>	6.0 x 10 <sup>2</sup>
	REVCO (-70°C)				
Spore Suspensions in Dimethyl Sulfoxide	Liquid Nitrogen	1.2 x 10 <sup>4</sup>	5.6 x 10 <sup>3</sup>	1.4 x 10 <sup>4</sup>	6.4 x 10 <sup>3</sup>
	Liquid Nitrogen				
Spore Suspensions in Dimethyl Sulfoxide	Liquid Nitrogen	1.5 x 10 <sup>4</sup>	6.2 x 10 <sup>3</sup>	1.1 x 10 <sup>4</sup>	5.0 x 10 <sup>3</sup>
	REVCO (-70°C)				
Spore Suspensions in Glycerine-Egg Yolk	Liquid Nitrogen	1.4 x 10 <sup>4</sup>	5.4 x 10 <sup>3</sup>	1.0 x 10 <sup>4</sup>	4.5 x 10 <sup>3</sup>
	Liquid Nitrogen				
Spore Suspensions in Glycerine-Egg Yolk	Liquid Nitrogen	1.4 x 10 <sup>4</sup>	9.0 x 10 <sup>2</sup>	2.0 x 10 <sup>4</sup>	2.0 x 10 <sup>3</sup>
	REVCO (-70°C)				

IC of fresh spores = 6 x 10<sup>3</sup>  
50

Results: Third stage larvae were apparently refractory to infection by spore suspension. Twenty-five percent of first stage larva exposed to spore suspension became infected. Four percent of third stage larvae became infected, when they were allowed to feed on infected A. aegypti larvae. The spore concentration required to infect 25 percent of first stage Toxorhynchites larvae was 116 times that required to infect 50 percent of 48 hr. old A. aegypti larvae.

#### 8. Survey of Sylvatic Rodents for Serological Evidence of Rabies Virus Infection

OBJECTIVE: To capture sylvatic rodents in selected forested areas of Thailand and to test their serum for rabies neutralizing antibodies.

BACKGROUND: On several occasions investigators at SEATO Medical Research Laboratory have examined wild rodents to determine if they were infected with rabies virus. In the first reported survey, Smith, et al., 1967-68, rabies virus was found in six different species of rodents in Thailand (10,11). Subsequent surveys have yielded negative results. In the Annual Report of 1971, Hickman, et al., reported that negative results obtained during the four year period since rodents were implicated as a possible sylvatic reservoir of rabies virus infection, suggests that the hypothesis of a rodent reservoir of rabies in Thailand is false. In all of these surveys the basis for evaluating rodents for rabies virus infection was by examination of brain tissue using a fluorescent antibody test. Positive specimens were confirmed by intracerebral inoculation of weanling mice.

In this study we propose to screen select wild rodent populations for evidence of exposure to rabies virus by testing their serum for rabies neutralizing antibodies. Similar serological monitoring procedures has been used by other investigators to determine the incidence of rabies in a wildlife population. There are no records which indicate this type of survey has been performed in Thailand.

Serological test results will provide useful information. If, as recent surveys indicate, there is no evidence of rabies infection, one would favor the conclusion that rabies is not an endemic disease of sylvatic rodents in Thailand, and that the initial findings reported by Smith, et al., were false. However, positive serology would lend credence to the initial findings and suggest that rabies or a rabies-like virus is an endemic disease of rodents in Thailand.

TABLE 1  
SUMMARY OF RODENT SPECIES TRAPPED

Genus Species	Location	
	Sakaraj	Doi Suthep
<u>Rattus sabanus</u>	6	
<u>Menetes berdmorei</u>	1	4
<u>Tupia glis</u>	17	1
<u>Rattus surifer</u>	80	
<u>Rattus koratensis</u>	1	
<u>Rattus rattus</u>	13	5
<u>Rattus fulvescens</u>	8	
Civet cat	1	
<u>Bandicoota indicus</u>		6
<u>Rattus exulans</u>		1
Moongoose		1
<u>Rattus bukit</u>		1

METHODS: Rodents were trapped in forested areas using SMRL live animal traps. All trapping was coordinated with the Thai Forestry Division and entailed field trips of 4 to 5 days duration at each site.

Each rodent was assigned an identifying number, typed by genus, species, sex, age, and the trapping site was identified. Separately caged, the rodents were transported to the Department of Veterinary Medicine for additional examination and bleeding. During collection of blood, rodents inadvertently killed were stored at -60°C until test results became available. Serum was tested for rabies neutralizing antibodies using the mouse test. A titer of 1:5 or greater will be considered indicative of exposure to rabies or rabies-like virus infection. Rodents serologically negative are killed. Any rodents with serological evidence of rabies virus infection are saved for additional study.

RESULTS: The study is in progress and only initial results are available. At the Sakaraj Scientific Research Center a total of 127 rodents have been trapped (Table 1). Limited trapping at Doi Suthep in Chiangmai yielded 19 rodents (Table 1). Serological testing is in process.

#### 9. Animal Rabies in Thailand: Rabies Diagnostic Laboratory Services

OBJECTIVE: To provide rabies diagnostic services to U.S. military personnel in Southeast Asia and the Western Pacific.

METHODS: Every brain submitted was examined by the fluorescent antibody test and confirmed by mouse inoculation. (12)

RESULTS: Of 521 brain specimens examined, 221 (42%) were positive (Table 1). Prevalence of rabies in dogs was 48% and in cats 9%.

Following the withdrawal of most U.S. armed forces in July 1976, receipt of rabies specimens from authentic U.S. military sources virtually ceased. In CY 1977, less than one specimen per month has been submitted by a U.S. governmental agency. Presently, practically all work in the rabies laboratory is in support of research activities. Unless circumstances change, this will be the last report of rabies diagnostic laboratory services.

<u>Species</u>	<u>Number of Specimen</u>	<u>Number Positive</u>	<u>Percent Positive</u>
Canine	446	215	48
Feline	44	4	9
Non-human Primate	8	0	0
Bat	2	0	0
Rodent	16	0	0
Porcine	1	0	0
Human	1	1	100
Bovine	2	1	50
Ovine	1	0	0
Total	521	221	42

10. A Survey for Viral Agents Transmitted by Culicoides in Northern Thailand

OBJECTIVE: To isolate and identify viral disease agents from Culicoides collected in Chiangmai Valley.

BACKGROUND: Studies in 5 provinces of Northern Thailand-- Chiangmai, Chiang Rai, Phrae, Lampang and Mae Hong Sorn-- revealed the presence of 35 species of blood sucking midges, including one species of daytime-biting midges, Forcipomyia anabaenae, 33 species of Culicoides and Leptoconops xuthoscelis(13). Although Culicoides midges have not yet been implicated in the transmission of arboviruses in Thailand, a total of 20 viruses have been reported recovered from wild-caught Culicoides elsewhere in the world (14). Interest in the vector potential of these biting midges in Northern Thailand arises as the result of the reported isolation of Ingwavuma virus in the Chiangmai Valley in 1970 (15).

METHODS: Culicoides were collected in CDC light traps (with and without CO<sub>2</sub> as an attractant) which were placed in rural villages located in Doi Saket, Hang Dong, Mae Rim and Sarapee

Districts of the Chiangmai Valley. Collections were also made by aspiration of midges attracted to cattle and buffalo.

Specimens were killed by freezing on dry ice and returned in frozen state to the lab in Bangkok and stored at -70°C. After identification and pooling by species, the pools will be processed for virus isolation attempts.

RESULTS: Collections using CDC light traps with CO<sub>2</sub> were made on 53 nights between 17 March and 1 September. Light trap collections without CO<sub>2</sub> were made on 18 nights, with biting and resting collections in animal pens being made on the same nights. Rainfall was quite low during the months of March thru July and may have contributed to the reduced population of Culicoides in the study area. Consequently only low numbers of adults were collected in all areas. Identification and virus-isolation results are not yet complete.

#### 11. Leukocyte Subpopulations in Patients Infected with Dengue Hemorrhagic Fever

OBJECTIVE: To characterize circulating blood lymphocytes of dengue patients during the acute stage of infection and in convalescence.

BACKGROUND: The host immune response to viral infections is characterized by its complexity. This response involves both cellular and humoral components as well as interferon synthesis (16). As one aspect of earlier studies, the cellular immunology of viral infections has been extensively studied and reported (17). Although the amount of effort in this research area has been considerable, there is a paucity of information pertaining to patterns of circulating leukocytes of the host. The investigation of such phenomena serves as the basis of this report.

METHODS: Blood specimens were obtained from juveniles admitted to Children's Hospital, Bangkok, Thailand. Infections were confirmed by serodiagnosis. Mononuclear leukocytes were isolated according to the method of Boyum (18). Rosetting procedures were conducted according to the methodology of Mendes et al (19) with modification (20). Briefly, the percentage of thymic dependent (t) lymphocytes were determined by sheep red cell rosetting with incubation times of 5 minutes, 1 hour and 18 hours. The percentages of Fc receptor positive cells and thymic independent (B) lymphocytes were likewise determined by

Table 1. Lymphocyte Rosette Values of Dengue Patients

Patient Status	E Rosette Cells (%)			EA Rosette Cells (%)	EAC Rosette Cells (%)
	5 minute incubation	1 hour incubation	18 hour incubation		
Acute	Range	21-41	25-46	31-60	5-16
	Mean	32	37	46	10
	S.D.	$\pm 5.3$	$\pm 6.7$	$\pm 8.1$	$\pm 3.0$
Convalescent (15 days)	Range	33-52	41-58	50-71	4-25
	Mean	44	51	63	12
	S.D.	$\pm 6.3$	$\pm 5.7$	$\pm 4.9$	$\pm 5.3$
Convalescent (30 days)	Range	32-60	45-63	63-73	6-15
	Mean	46	58	67	9
	S.D.	$\pm 6.2$	$\pm 6.1$	$\pm 3.1$	$\pm 6.0$

Table 2. Circulating Lymphocyte Subpopulations in Dengue Patients

Stage	WBC per mm <sup>3</sup>	Lymphocytes per mm <sup>3</sup>	E Rosette Cells			EA Rosette Cells	EAC Rosette Cells
			5 minute	1 hour	18 hour		
Acute	8816	4461	1406	1643	2022	403	730
Day 15	10147	4036	1766	2034	2528	455	677
Day 30	8713	3955	1879	2194	2861	379	738

rosetting. Leukocyte populations were also monitored by differential counts and the projected concentrations of leukocyte subclasses were calculated.

RESULTS: Table 1 summarizes the lymphocyte rosette data of the 17 dengue patients who participated in this study. All T lymphocyte patterns were similar. Regardless of the incubation time, the mean value indicated suppression in the acute stage of infection followed by increasing values with convalescence. The values for the Fc cells were fluctuant and while differences were slight there was a suggestion of enhancement during the acute and early convalescent stages. The means for the B lymphocytes were essentially unchanged for the duration of the assay. The projected numbers of circulating lymphocytes are summarized in Table 2. While most values appeared to be stable, those for the E rosette cells (T lymphocytes) are especially noteworthy by their suppression. It is hypothesized that the considerable proportion (mean 10%) of atypical lymphocytes circulating during the acute phase may be, in part, T lymphocytes with impaired ability to form E rosettes. A manuscript on this work is in preparation. This is a final report.

#### 12. Isolation of Dengue Virus from Patients in Provincial Hospitals

OBJECTIVE: To isolate dengue viruses from patients seen at provincial hospitals and compare the virus serotypes with those isolated in Bangkok.

BACKGROUND: Since 1962 the Medical Research Laboratory has isolated dengue viruses from patients at the Children's Hospital in Bangkok. Through this period several shifts in the dengue types occurring in the city have been documented. Dengue 2 virus has been present in Bangkok each year that isolations were attempted. In the early 1960's dengue 1, 3 and 4 were also present. Dengue 4 was isolated in 1965 but from 1969 through 1975 it was not found in Bangkok patients. From 1972 through 1975, dengue 1 and 3 made up approximately 50% of the isolates with dengue 2 making up the rest. In 1976 dengue 1 and 3 appeared to have been completely replaced by dengue 4.

During 1977 the incidence of dengue infections throughout Thailand increased earlier than expected and to unprecedented levels. Possibly this increase represents the reintroduction of Dengue 4 throughout the country.

TABLE 1  
Virus Isolations from Patients Admitted to  
Provincial Medical Centers in Thailand

Provincial Hospital	Number Studied	Isolation No.	%
Phrae	23	7	(30.4)
Khon Kaen	42	14	(33.3)
Ubon	34	4	(11.8)
Udorn	24	10	(41.7)
Total	123	35	(28.5)

Study of the history of dengue infection in Thailand suggests that virus transmission has been endemic in Bangkok since the late 1950's if not before. Throughout the years sporadic outbreaks of dengue occurred in the provincial areas, possibly representing introduction of dengue virus into these areas from Bangkok. Over the past several years dengue infection has been occurring regularly in certain provincial centers; e.g., Ubon, Udorn, Pitsanuloke, Khon Khaen, and Chanthaburi. It has been suggested that in these areas dengue may have become endemic. The Laboratory has not attempted isolation from upcountry provincial areas for many years. The purpose of this study was to isolate dengue virus from patients seen in provincial hospitals and to compare the virus serotypes to those isolated in Bangkok.

METHODS: Following consultation with Dr. Sujarti, collection teams were placed in provincial hospitals found to be admitting large numbers of patients with clinical dengue hemorrhagic fever (DHF). These teams, with the help of the director and the pediatric staff of the hospital, collected bloods on patients with signs and symptoms of dengue infections. Acute serum and heparinized plasma were obtained on patients who had been clinically ill for four days or less. A convalescent serum was then collected two to five days later. A short history and physical examination was recorded on each patient.

Serum and plasma samples were stored in liquid nitrogen and transported to the laboratory. Routine virus isolation was carried out on acute plasma using a direct and delayed plaque technique on LLC-Mk<sub>2</sub> cells. Viruses were identified by a plaque reduction neutralization technique using monkey antisera made to prototype dengue virus serotypes.

RESULTS: At the time of this report, 35 viruses have been isolated from specimens collected from four provincial medical centers. (Table 1) Identification of the virus isolated, serology on the patients collected and integration of this data with the historical and demographic information collected will be undertaken.

#### 13. A Serological Survey for Togaviruses (Arboviruses) in a Well Defined Rural Thai Population

OBJECTIVE: To study the seroepidemiology of Togavirus (arbovirus) infections in a well defined rural Thai population.

BACKGROUND: Dengue virus was first specifically identified as a cause of illness in Thailand in the early 1950's. At that time,

Prevalence of Togavirus Antibodies in a Well Defined  
Thai Rural Population

Age	No. Tested	Alpha Virus <sup>a</sup>		Flavivirus <sup>b</sup>	
		No.	(%)	No.	(%)
1	5			1	(20.0)
2	15			4	(26.0)
3	23			6	(21.0)
4	37	2	(5.4)	33	(89.2)
5	21			17	(80.9)
6	35	2	(5.7)	29	(82.9)
7	35	4	(11.4)	30	(85.7)
8	24	1	(4.1)	17	(70.8)
9	24	3	(8.3)	20	(83.3)
10-14	54	13	(24.0)	47	(87.0)
15-19	52	6	(11.5)	45	(86.5)
20-29	92	22	(23.9)	85	(92.4)
30-39	55	20	(36.4)	541	(98.2)
40-49	33	18	(54.5)	33	(100.0)
50	22	12	(54.5)	20	(90.9)
	526	103	(19.6)	441	(83.8)

a Chikungunya

b Dengue 1-4 and Japanese encephalitis

and for many years thereafter, it was felt that dengue infections were endemic only in the large cities. More recently, it has been recognized that dengue infections also occur in rural populations. However, little is known of the prevalence of arbovirus antibody in rural Thailand, as most studies done outside of cities involved areas of epidemic illness. As sera were already being collected from village populations for malaria and hepatitis studies, a serological survey for arbovirus infection was included.

MATERIALS & METHODS: The materials and methods for this study are outlined elsewhere in this report (the Epidemiology of Hepatitis B Virus in a Well Defined Rural Population). Serum was submitted for routine hemagglutination inhibition tests using an alpha virus (arbovirus group A) antigen, chikungunya (chik) and flavivirus (group B arbovirus) antigens, dengue 1-4 (DEN 1-4) and Japanese encephalitis (JE).

RESULTS: Of a total population of 1041 people in the village, sera from 526 had been tested by the time of this report. Of these, 230 sera were obtained from people from age 1 to 10. This age group made up over 60% of the village population. The prevalence of antibody to chikungunya in the whole population was 19.2%; it was 6.1% in the tested children ten years old or less and rose to 54.5% by the 40-49 year age group, remaining at that level in the few older persons studied. The flavivirus antibody, on the other hand, rose rapidly, starting at 20% in the few one year olds bled but reaching 89.2% by the age of four years and remaining over 80% for all but one of the remaining age groups.

This data serves as a good comparison to that collected in Bangkok in 1962 and 1977 and illustrates that flaviviruses transmission is much more rapid in rural environments than in Bangkok. The antibody prevalences to flaviviruses are very similar to those found in Phnompenh in 1974 (21).

The collection of materials from the village of Tablan is finished. This report presents preliminary data as the laboratory tests are still underway.

14. A Longitudinal Epidemiological Study of Dengue Virus Infections in a School Population

OBJECTIVE: To conduct a longitudinal study of dengue virus infections in a lower socioeconomic school population.

BACKGROUND: Dengue hemorrhagic fever remains among the greatest known causes of hospitalization and death among children in Thailand. Examination of the age distribution of reported dengue cases admitted to hospitals over a ten year period has shown an increase in the median age of patients from three years ten months during the years 1962-1965 to five years and seven months in 1971-1973 (22). This suggested a change in the transmission rate of dengue viruses and possibly a change in the number and age of children susceptible to dengue infection.

A study of dengue antibody prevalence, conducted before and after the transmission season, was made in Bangkok in 1962 (23). At that time, there was a preseasional prevalence of Dengue 1 antibody of approximately 60% in the 4-15 year age groups drawn from a mixed socioeconomic population. The prevalence rose from approximately 40% in the four year olds to 80% at 15 years of age.

Based upon 1962 studies, a hypothesis was developed relating severity of dengue infection to a second exposure to dengue viruses. This has come to be called the secondary exposure hypothesis. This hypothesis is now being questioned as it does not fully explain the findings of severe disease in patients with no evidence of prior dengue infection (24). A study was undertaken to examine the epidemiology of dengue fever in a lower socioeconomic school population throughout a dengue transmission season to ascertain the relationship of severity to secondary infection.

METHODS: Students attending the Pibulprachasan School in the Dindaeng area of Bangkok were studied. Students were asked to obtain a signed consent form from their parents. All children volunteering for this study were bled prior to the dengue transmission season in May and June 1976. From May through September all children absent from classes for more than two days for physical reasons were visited by a public health nurse. A history of the student's disease was obtained from the family and blood samples were drawn from the student and from members of his family. Approximately 15 days after the initial bleeding, all concerned family members were bled again.

Hospital records were obtained on students whose illness led to hospitalization.

TABLE 1. Population Structure of School Population that was selected for  
Dengue Virus Epidemiology Studies

Age	Male		Female		Total	
	No.	(%) <sup>a</sup>	No.	(%)	No.	(%)
4	15	(0.7)	33	(1.7)	48	(2.4)
5	29	(1.5)	33	(1.7)	62	(3.1)
6	56	(2.8)	35	(1.8)	91	(4.6)
7	85	(4.3)	103	(5.2)	188	(9.5)
8	115	(5.8)	88	(4.4)	203	(10.2)
9	116	(5.8)	110	(5.5)	226	(11.4)
10	123	(6.2)	127	(6.4)	250	(12.6)
11	108	(5.4)	107	(5.4)	215	(10.8)
12	115	(5.8)	131	(6.6)	246	(12.3)
13	111	(5.6)	168	(8.5)	279	(14.0)
14	42	(2.1)	79	(4.0)	121	(6.1)
15	18	(0.9)	28	(1.4)	46	(2.3)
Total	933	(47.2)	1042	(52.9)	1975	(100)

a Percent of total subjects tested

TABLE 2. Dengue Virus Antibody Prevalence Rates Determined for Students Prior to the Dengue Virus Epidemic Season

Age	Males		Females		Number Tested	Dengue Virus Antibody (%) <sup>a</sup>	Dengue Virus Antibody No.	(12.0)	48	9	(18.8)
	Number Tested	Dengue Virus No.	Number Tested	Dengue Virus Antibody (%) <sup>a</sup>							
4	15	5 (33.3)	33	4 (12.0)							
5	29	14 (50.0)	33	13 (39.4)							
6	56	20 (35.7)	35	16 (45.7)							
7	85	35 (41.2)	103	41 (39.8)							
8	115	61 (53.0)	88	38 (43.2)							
9	116	64 (55.1)	110	59 (53.6)							
10	123	75 (61.0)	127	68 (53.5)							
11	108	55 (51.0)	107	54 (50.5)							
12	115	71 (61.7)	131	73 (55.7)							
13	111	74 (66.7)	168	114 (67.9)							
14	42	30 (71.4)	79	49 (62.0)							
15	18	16 (88.9)	29	15 (51.7)							
Total	933	520 (55.7)	1042	546 (52.4)	1975					1064	(53.9)

<sup>a</sup> Percent of number tested

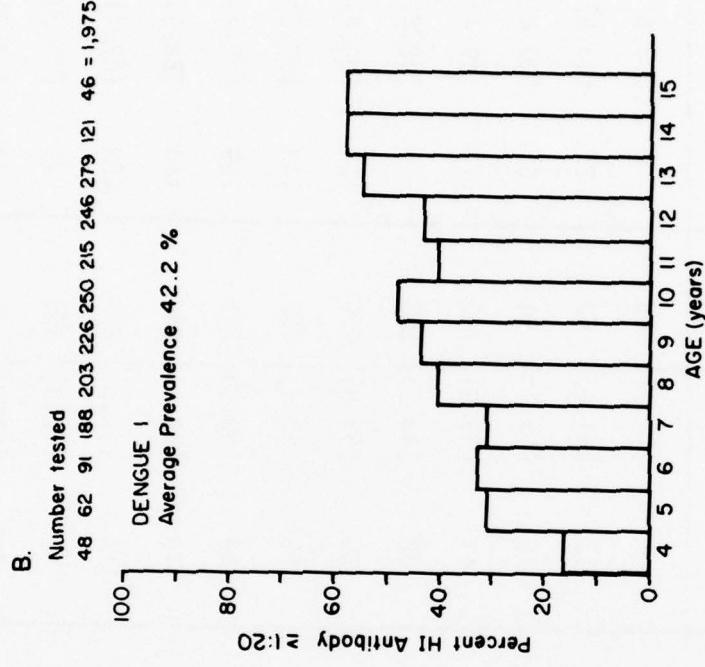
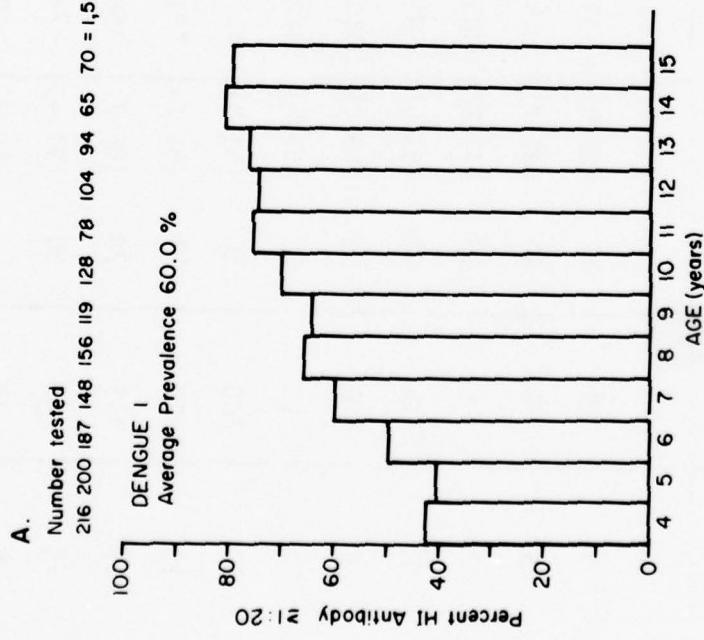


Figure 1. Frequency of occurrence of dengue - I HI antibody by age in Bangkok Sample Populations

A. "Pre season" 1962 bleed from Bangkok area sample, taken from Halstead, S. B., et al. Am. J. Trop. Med. Hyg [8]:1009

B. "Pre season" 1977 bleed from Pibulprachassan School, Bangkok, THAILAND.

Sera was submitted for hemagglutination inhibition testing against dengue 1-4, Japanese encephalitis and chikungunya antigens using standard methods.

RESULTS: Blood samples were drawn from 1975 children, 933 males and 1042 females between the ages of four and 15 years (Table 1). The prevalence of antibody to any of the four dengue types was 54% (Table 2). The prevalence rose from 19% in the four year age group to 67% by age 15.

We have prepared a histogram of the prevalence of dengue 1 antibody by year of age in 1977 (Figure 1A) in order to compare this data with that reported from the 1962 study (Figure 1B).

The shape of these two histograms is different and the average percent with antibody was 60% in 1962 compared with 42% in 1977. Of the 1975 children studied this year, chikungunya antibody was found in 14.5% and Japanese encephalitis antibody alone was detected in 28 (1.4%). In the 23 absentees adequately studied at this time, only 2 required hospital out-patient care. These 2 patients had no serological evidence of dengue virus infection but both of them had a four-fold serological rise to chikungunya. There were seven patients exhibiting four-fold rises to dengue virus. None of these seven required hospital care; they all had low antibody titers indicative of probable primary infection. There have been no secondary infections (as defined by HI criteria) detected in the students so far studied.

We have presented early and preliminary data on the dengue epidemiology seen in a Bangkok school population. Post transmission season bleeds will be collected in December 1977 and March 1978. Longitudinal dengue antibody data should give some indication of the incidence of clinical and subclinical dengue in this group as well as possibly elucidate the severity of primary and secondary disease.

#### 15. Isolation of Viruses from Leukocytes of Dengue Patients

OBJECTIVE: To determine if dengue virus can be isolated from leukocytes during natural dengue infections and to identify the cells infected.

BACKGROUND: Dengue viruses have classically been isolated from human serum or plasma. Studies on the pathogenesis of dengue virus infections in man and monkeys suggested that these viruses may also be associated with the formed elements of the blood.

That peripheral blood leukocytes might be a source of virus has been shown by Marchette et al. (25) in dengue infected Rhesus monkeys. In man, fluorescent antibody studies by Boonpucknavig et al. have identified leukocyte associated dengue antigens (26). Also, several laboratories have demonstrated the in vitro replication of dengue viruses in varying types of human leukocyte cultures (27, 28, 29 and 30). These observations indicated that isolation of viruses from the leukocytes of dengue patients might be rewarding.

METHODS: Clinical histories and blood samples were collected from patients admitted to the Bangkok Children's Hospital. The first day of fever was defined as the first day of clinical illness. Two blood samples were collected on the day of admission and approximately 15 days later.

The severity of illness was graded using the following criteria.

Grade I: Fever accompanied by non-specific constitutional symptoms; the only hemorrhagic manifestation is a positive tourniquet test.

Grade II: Fever accompanied by skin hemorrhage or other bleeding such as from the nose or gums.

Grade III: Circulatory failure manifested by rapid, weak pulse, narrowing of pulse pressure ( $\leq$  20 mm Hg) or hypotension.

Grade IV: Blood pressure and pulse are undetectable.

Grades I and II were considered dengue hemorrhagic fever without shock and grades III and IV were dengue shock syndrome. Sera obtained from each individual were tested simultaneously for antibodies by hemagglutination inhibition tests.

Each case was classified as either primary or secondary dengue infection. Patients with convalescent titers less than 1:640 to three or more dengue types were assumed to have primary infections. Those with convalescent titers of 1:640 or greater to two or more dengue antigens were considered to have secondary infections.

A dextran sedimentation method was used to separate the formed components of the blood. Heparinized blood was divided into cell free plasma and a cellular pellet by centrifugation. The pellet was resuspended in Dextran T-250 and the red blood cells were allowed to sediment and were discarded. The supernatant was centrifuged at 150 x g to sediment the leukocytes. Viruses were isolated using a direct and/or delayed plaque technique on LLC-Mk<sub>2</sub> cells depending on the sample. In some cases, aliquots of leukocyte suspensions were transferred to tissue culture flasks. Following incubation, nonadherent cells were removed and adherent cells were vigorously washed. Both types of cells were assayed for virus by a delayed plaque technique. Isolates were confirmed and identified by a plaque reduction neutralization test using monkey antisera prepared against prototype dengue strains.

RESULTS: Isolation from plasma and leukocytes were attempted on 211 patients who were adequately followed and had a clinical picture of dengue infection. Six of these had serological evidence of chikungunya and one patient yielded chikungunya virus from both plasma and leukocytes. Serological evidence of dengue infection was found in 195 of these patients; 47 of them yielded virus. To date, we have identified 32 dengue strains, 14 dengue type 4 and 18 dengue type 2. The 47 viruses were composed of one isolate from plasma alone, 15 isolates from plasma and leukocytes and 31 isolates from leukocytes alone (Table 1). The use of leukocytes allowed over three times the recovery rate compared to that obtained from plasma.

The number and percent of isolates by day of clinical illness are shown in Table 2. Samples obtained early in the courses of disease were most likely to yield virus. Plasma served as a source of virus in 8.2% of patients as compared to 23.5% for leukocytes. Plasma yielded virus isolations during the first four days of disease while virus could be recovered from leukocytes through the sixth day of disease. Furthermore in samples collected during the first four days, plasma yielded virus in only 22% in contrast to leukocytes which yielded almost 50%. We broke down the 195 patients with serological evidence of dengue infection by age, sex, primary or secondary infection and severity of disease (Table 3, 4). There was no apparent relationship between dengue isolation and sex or age group with the exception that viruses were more frequently recovered in older children.

There were 14 patients with primary dengue infection. The isolations from these primary patients by severity of illness are illustrated in Table 5. Three of these primary patients were

Table 1.  
Virus Isolations from 195 Dengue Patients  
Bangkok 1976 - 1977

Specimen	Number	Percent of Isolations
Plasma	1	2.1
Leukocytes	31	66.0
Both	15	31.9
Total	47	100.0

1004

Table 2.  
Isolation of Viruses from Human Plasma and  
Leukocytes during Dengue Infections

Day of <sup>a</sup> Disease	Patients Studied	Positive Specimens					
		Plasma		Leukocytes		Both	
		No.	%	No.	%	No.	%
2	3	1	33.3	1	33.3	1	33.3
3	21	9	42.9	15	71.4	16	76.2
4	49	6	12.2	18	36.7	18	36.7
5	57	0	0	10	17.5	10	17.5
6	31	0	0	2	6.5	.2	6.5
7-10	34	0	0	0	0	0	0
Total	195	16	8.2	46	23.6	47	24.1

<sup>a</sup> Day after first day of fever

Table 3  
Dengue Patients by Grade of Severity and  
Sequence of Infection

Grade of Severity	Sequence of Dengue Infections		
	Primary	Secondary	Total Infections
UF <sup>a</sup>	7 (50.0) <sup>b</sup>	7 (3.9)	14 (7.2)
I	0 (0)	6 (3.3)	6 (3.1)
II	4 (28.5)	69 (38.1)	73 (37.4)
III	3 (21.4)	83 (45.9)	86 (44.1)
IV	0 (0)	16 (8.8)	16 (8.2)
Total	14	181	195

a Undifferentiated fever

b Percent of total in primary or secondary group

Table 4.  
Virus Isolations from Leukocytes by Age and Sex  
Bangkok Children's Hospital, 1976 - 1977

Age	Male			Female			Total		
	No.	Isolations		No.	Isolations		No.	Isolations	
		Tested	No.		Tested	No.		No.	%
2	4	0	0	9	2	22.2	13	2	33.3
3	9	2	22.2	8	2	40.0	17	4	23.5
4	9	0	0	9	2	22.2	18	2	71.1
5	9	0	0	8	0	0	17	0	0
6	8	1	12.5	15	4	26.6	23	5	21.7
7	9	1	11.1	9	1	11.1	18	2	11.1
8	4	0	0	10	1	10.0	14	1	7.1
9	6	1	16.7	12	5	41.7	18	6	33.3
10	7	2	28.5	10	5	50.0	17	7	41.2
11	5	3	60.0	7	1	14.3	12	4	33.3
12	8	5	62.5	9	3	33.3	17	8	47.0
13	5	3	60.0	4	2	50.0	9	5	55.5
14	1	0	0	0	0	0	1	0	0
15	1	0	0	0	0	0	1	0	0
Total	85	18	21.2	110	28	25.5	195	46	23.6

Table 5.  
The Relationship of Severity of Disease to the Virus Isolation Rate  
in Primary Dengue Cases, Bangkok 1976-1977

Grade of Severity	Number studied	Positive Specimens			Both No. (%)
		Plasma No.	(%) <sup>b</sup>	Leukocytes No.	(%)
UF <sup>a</sup>	7	1	(14.3)	2	(28.6)
I	0				
II	4	1	(25.0)	2	(50.0)
III	3	1	(33.3)	1	(33.3)
IV	0				
Total	14	3	(21.4)	5	(35.7)
				5	(35.7)

a UF = Undifferentiated fever

b Percent of number studied

Table 6.  
The Relationship of Severity of Disease to the Virus Isolation  
Rate in Secondary Dengue Cases, Bangkok 1976 - 1977

Grade of Severity	Number studied	Positive Specimens			Both No. (%)
		Plasma No.	(%) <sup>a</sup>	Leukocytes No.	
UF <sup>a</sup>	7	0		1 (14.3)	1 (14.3)
I	6	2	(33.3)	1 (16.7)	2 (33.3)
II	69	6	(8.7)	15 (21.7)	15 (21.7)
III	83	4	(4.8)	21 (25.3)	21 (25.3)
IV	16	1	(6.3)	3 (18.8)	3 (18.8)
Total	181	13	(7.3)	41 (22.9)	42 (23.5)

a UF = Undifferentiated fever

b Percent of number studied

Table 7.  
Recovery of Virus from Adherent and Non-Adherent Leukocytes

Leukocytes	Isolations	
	No.	(%)
Adherent	10	(55.5)
Adherent & Non-Adherent	6	(33.3)
Non Adherent	2	(11.1)
Total	18	(100.0)

1010

older children of grade III severity. Due to later hospital admission, virus was recovered from the leukocytes of only 35% of the patients. Because of the small number of isolates, patterns were difficult to discern.

Table 6 shows the virus isolation rates and the severity of disease in 181 patients with secondary dengue infection. Here there appeared to be no relationship between the virus isolation rates and the severity of disease.

We have begun to identify the cells which are infected with viruses. Isolations from adherent and non-adherent cells of 18 patients are presented in Table 7. Virus was obtained from adherent cells in ten patients, from adherent and non-adherent cells in two patients. This suggests that the phagocytic monocyte might be the site of virus infection; however, recovery of virus from non-adherent cells indicates that virus might be associated with other white blood cells as well. Identification of the leukocytes infected with dengue virus *in vitro* is continuing.

#### 16. Evaluation of *Toxorhynchites splenens* as a Bioassay Host for Isolating Dengue Viruses

OBJECTIVE: To evaluate *Toxorhynchites splenens* as a bioassay host for detecting and replicating dengue viruses.

BACKGROUND: Investigations employing a laboratory colony of *Aedes aegypti* as a bioassay host for dengue viruses have yielded inconsistent results. Available data suggested that the susceptibility to dengue virus infection varied for individual mosquitoes. Pertinent findings include the failure to detect dengue viruses in all *A. aegypti* that were inoculated with  $10^6.0$  PFU/0.3 ml following a 14 day incubation period. Variation in susceptibility was observed more frequently for dengue 1, 3 and 4 than for dengue 2 virus. In addition, the low infectivity yields associated with mosquitoes after being inoculated with high concentrations of dengue viruses and with dengue virus infected leukocytes failed to provide evidence of virus amplification. These findings were based on a plaque assay, complement fixation and fluorescent antibody assay of mosquitoes. This report consists of the preliminary findings of an investigation initiated to evaluate the mosquito bioassay system employing *T. splenens*.

MATERIALS & METHODS: Mosquitoes were obtained from a laboratory colony of *T. splenens* that was established during July 1976 in the Department of Entomology, AFRIMS. *Culex quinquefasciatus*

larvae were provided continuously to T. splenens larvae and the diet for the adults consisted of honey. Dengue viruses employed were mouse seeds including type 1, (Hawaiian strain, 16th passage), type 2, (New Guinea c, 29th passage), type 3, (H87, 25th passage), and type 4, (H241, 31st passage). The infectivity titer in LLC-Mk<sub>2</sub> cells were  $1.5 \times 10^{6.0}$ ,  $1.9 \times 10^{6.0}$ ,  $3.1 \times 10^{6.0}$  and  $1.7 \times 10^{6.0}$  PFU per 0.3 ml for dengue virus type 1, 2, 3 and 4 respectively.

Mosquitoes were inoculated with  $\log_{10}$  dilutions of each dengue virus serotype as described by Rosen and Gubler (31). Virus dilutions were prepared in RPMI 1640 medium, and the volume inoculated was 0.85  $\mu$ l per mosquito. After a 14 day incubation period at 32°C, mosquitoes were sacrificed for virus assay employing fluorescent antibody (FA) and complement fixation (CF) tests. The FA and CF tests were performed according to methods of Kuberski and Rosen (32, 33). Pooled human dengue virus antisera were labeled with fluorescent isothiocyanate (FITC) as described in the 1975-1976 SEATO Annual Progress Report. Tissue imprints resulting from squashing mosquito heads were flooded with FITC labeled antisera and examined with the 10x objective of a Leitz fluorescent microscope. The CF antigens were prepared by placing 2 T. splenens (thorax-abdomen) in 1.0 ml of chilled buffered saline and disintegrating them with sonic energy employing a S110 model sonifier. The suspension was centrifuged at 3,300 rpm for 15 minutes at 4°C, and the supernatant fluid was used as the CF antigen. Dengue virus hyperimmune antisera used in CF tests were prepared according to methods of Brandt et al (34). Uninfected mosquitoes were employed as controls in both the FA and CF test. FA observations were made by 2 or more observers.

RESULTS: The data of this study indicate that T. splenens was susceptible to infection on parenteral inoculation with dengue viruses. Susceptibility to infection appeared to be lower for dengue 3 and 4 viruses, especially the latter. In addition, the intensity of fluorescence and the CF titers were less for mosquitoes inoculated with dengue 3 and 4 viruses. Similar results have been reported for Aedes albopictus and dengue virus (33).

The low CF titers associated with dengue 4 inoculated mosquitoes that failed to yield positive FA results cannot be explained on the basis of data obtained in this study. Evidence based, however, on the low CF titers (1:4 to 1:8) observed for uninfected control

Table 1. Specific Fluorescence Detected in Head Tissue of Toxorhynchites splendens on Day 14 Following Inoculation with Log<sub>10</sub> Dilutions of Dengue Viruses

Virus	Dilution of Virus Inoculum					
	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
Dengue-1	2/3 <sup>a</sup>	4/4	4/4	4/4	1/4	0/4
Dengue-2	4/4	4/4	4/4	4/4	2/4	0/4
Dengue-3	0/2	2/4	4/4	2/4	3/4	0/4
Dengue-8	1/3	3/3	3/3	2/2	1/3	0/3
Dengue-4	2/4	1/4	0/4	0/4	0/4	-
Dengue-4	0/3	0/4	2/4	2/4	1/3	0/4
Controls	0/6	0/6	0/6	0/6	0/6	0/6

a No. fluorescence positive/no. fluorescence negative mosquitoes  
 b Not tested

Table 2. Complement Fixing Titors of Dengue Virus Hyperimmune Antisera with Antigens Prepared from Toxorhynchites splenens Infected with Dengue Viruses

Virus	Antigen Dilution of Inoculum	Dengue Virus Antisera			
		D-1	D-2	D-3	D-4
Dengue-1	$10^{-1}$	512 <sup>a</sup>	128	16	0
	$10^{-2}$	1024	256	8	0
	$10^{-3}$	512	8	8	0
	$10^{-4}$	512	32	32	0
	$10^{-5}$	2048	256	16	0
Dengue-2	$10^{-1}$	128	256	16	32
	$10^{-3}$	64	256	16	4
	$10^{-5}$	128	1024	32	8
Dengue-3	$10^{-1}$	32	32	16	16
	$10^{-3}$	32	16	8	8
	$10^{-5}$	16	16	16	16
Dengue-3	$10^{-1}$	64	16	16	16
	$10^{-2}$	32	32	32	32
	$10^{-3}$	16	16	16	8
Dengue-4	$10^{-1}$	16	2	4	16
	2	8	8	2	16
	3	16	8	2	8
	4	8	8	2	4

a Reciprocal complement fixation titers

mosquitoes suggests that the mosquitoes may not have been infected. Further investigations employing infectivity assays in conjunction with FA tests will be required to address this question.

In regard to the use of CF tests for identifying dengue viruses, the data of the study indicate that dengue 3 and 4 viruses will require additional studies. Such studies will need to consider virus passage level and the specificity of the antisera.

Specific fluorescence was observed in head squashes of T. splenens following the inoculation of this mosquito with different concentrations of dengue 1, 2, 3 and 4 viruses, (Table 1). The most characteristic form of specific fluorescence was the appearance of rings of fluorescence which were observed in conjunction with well defined areas of background fluorescence. The amount of tissue exhibiting fluorescence and the intensity of fluorescence was substantially lower in mosquitoes inoculated with dengue 3 and 4 viruses. Non-specific fluorescence was observed in tissue smears prepared from control and infected mosquitoes. The more prevalent form was small fluorescent granules that appeared to be associated with the surface, as opposed to being within the mosquito tissue.

After removing the head of T. splenens for FA assay, the thorax and abdomen portions of the mosquito were employed as antigens for virus identification studies in CF tests. As shown in Table 2, dengue 1 and 2 viruses could be identified; however, this was not possible for dengue 3 and 4 viruses. CF titers for the latter viruses were also much lower than those observed for dengue 1 and 2 viruses. On the basis of data of this study and of previous findings mentioned regarding A. aegypti, the mosquito assay system cannot at this point be considered to be a reliable technique for isolating and identifying dengue viruses. Further studies to perfect the mosquito inoculation technique are underway.

17. The Influence of Different Cell Culture Media on the Infectivity Yield of Dengue Viruses

OBJECTIVE: To determine the effect of different cell culture media on the infectivity yields of dengue 1, 2, 3 and 4 viruses in LLC-Mk<sub>2</sub> cells.

BACKGROUND: Epidemiological, clinical and molecular investigations on dengue viruses continue to be complicated by low infectivity yields of these viruses in cell cultures and mice. The low yields are a major concern of the epidemiologist and clinician in regard

to the isolation and identification of dengue viruses. Even with a virus isolation, repeated passage using in vitro systems may not raise the infectivity titers sufficiently to allow for identification by the plaque reduction neutralization test.

Recent findings suggested that the yield of dengue 2 virus in LLC-Mk<sub>2</sub> cells was greater when the cells were maintained on Roswell Park Memorial Institute (RPMI) 1640 medium as opposed to Medium-199 (M-199). The influence of 1640 medium on the replication of other dengue viruses is unknown; no investigations have been conducted to determine if RPMI 1640 medium will increase the possibility of isolating and identifying dengue viruses obtained from naturally infected mosquitoes or humans.

METHODS: Dengue viruses 1, 3 and 4 were isolated from patient's plasma. Dengue 2 virus was isolated from a mosquito captured in the house of a dengue hemorrhagic fever patient. Dengue 2 virus (BM-50-76) was in the first LLC-Mk<sub>2</sub> passage while the other viruses (dengue-1, CH2624-74, dengue-3, 1337-74, and dengue-4, VN7-73) each received 10 passages in LLC-Mk<sub>2</sub> cells. LLC-Mk<sub>2</sub> cells were propagated in one ounce bottles employing Medium-199 supplemented with 15% calf serum. Confluent cell monolayers, free of medium, were inoculated with 0.2 ml aliquots of selected dilutions of each virus; the cultures were then incubated at 35°C for 2 hours. The cultures were replenished with the desired medium and placed at 35°C for 6 days. Media employed included M-199, Minimal Eagle's Media (MEM) and RPMI 1640 with and without fetal calf serum (FCS). On day seven, post inoculation cultures were subjected to a freeze-thaw cycle and then assayed for virus employing standard plaque techniques in LLC-Mk<sub>2</sub> cells.

RESULTS: Dengue virus infectivity titers in LLC-Mk<sub>2</sub> employing different media are shown in Tables 1 to 4. Except for dengue 2 virus, which appeared to replicate best in the presence of RPMI 1640 medium, no apparent difference in the infectivity yield was associated with different media. However, for dengue 1, 2 and 4, the dose of virus required to initiate infection appeared to vary depending on the medium used. In all cases when the lowest multiplicity of infection (MOI) was employed, virus was detected only in LLC-Mk<sub>2</sub> cultures maintained on RPMI 1640 medium. Also, the yield of virus appeared to be related to the MOI, that is, the optimum amount of inoculum tended to result in a satisfactory virus yield.

There were no marked differences in the infectivity yield of dengue virus in the presence of different media. The observation that minimal doses of dengue virus could initiate an

TABLE 1. Dengue Type 1<sup>a</sup> Virus Infectivity Yield in LLC-Mk<sub>2</sub>  
Maintained on Different Media

Inoculum (PFU/0.2 ml)	Media					
	M-199	M-199 + 5% FCS	MEM	MEM + 5% FCS	RPMI 1640	RPMI 1640 + 5% FCS
1x10 <sup>4</sup> (TNTC) <sup>b</sup>	4 x 10 <sup>3</sup> c	1.6x10 <sup>4</sup>	3.0 x 10 <sup>4</sup>	1.1 x 10 <sup>4</sup>	1.6 x 10 <sup>4</sup>	1.1 x 10 <sup>4</sup>
1x10 <sup>3</sup> (TNTC)	4 x 10 <sup>4</sup>	7.0x10 <sup>3</sup>	2.6 x 10 <sup>4</sup>	1.4 x 10 <sup>4</sup>	1.7 x 10 <sup>4</sup>	7.0 x 10 <sup>4</sup>
1x10 <sup>2</sup> (38)	7 x 10 <sup>3</sup>	9.0x10 <sup>2</sup>	1.0 x 10 <sup>4</sup>	3.0 x 10 <sup>3</sup>	1.2 x 10 <sup>4</sup>	7.0 x 10 <sup>3</sup>
1x10 <sup>1</sup> (9)	6 x 10 <sup>3</sup>	2.9x10 <sup>2</sup>	5.0 x 10 <sup>3</sup>	7.0 x 10 <sup>2</sup>	8.0 x 10 <sup>3</sup>	3.0 x 10 <sup>3</sup>
1 (1)	0 <sup>d</sup>	0	0	0	2.1 x 10 <sup>2</sup>	1.2 x 10 <sup>2</sup>
0	0	0	0	0	0	0

a. Dengue type 1, CH2624-74, Mk<sub>2</sub>-10

b. Actual count

c. PFU/0.2 ml of seeds harvested at day 7

d. Virus not detected

TABLE 2. Dengue Type 2<sup>a</sup> virus Infectivity Yield in LLC-Mk<sub>2</sub>  
Maintained on Different Media

Inoculum (PFU/0.2 ml)	Media				
	M-199	M-199 +5% FCS	MEM	MEM +5% FCS	RPMI 1640
2 x 10 <sup>4</sup> (TNTC) <sup>b</sup>	2 x 10 <sup>3</sup> c	6 x 10 <sup>3</sup>	3 x 10 <sup>3</sup>	4 x 10 <sup>3</sup>	8 x 10 <sup>5</sup>
2 x 10 <sup>3</sup> (TNTC)	4 x 10 <sup>3</sup>	6 x 10 <sup>3</sup>	3 x 10 <sup>3</sup>	6 x 10 <sup>3</sup>	1 x 10 <sup>6</sup>
2 x 10 <sup>2</sup> (24)	1 x 10 <sup>3</sup>	1 x 10 <sup>3</sup>	5 x 10 <sup>3</sup>	1 x 10 <sup>2</sup>	2 x 10 <sup>5</sup>
2 x 10 <sup>1</sup> (2)	3 x 10 <sup>2</sup>	1 x 10 <sup>2</sup>	8 x 10	1 x 10 <sup>2</sup>	5 x 10 <sup>4</sup>
2	0 <sup>d</sup>	0	0	0	5 x 10 <sup>3</sup>
0	0	0	0	0	0

a. Dengue Type 2, BM50-76, Mk<sub>2</sub>-1

b. Actual count

c. PFU/0.2 ml of seeds harvested at day 7

d. Virus not detected

TABLE 3. Dengue Type 3<sup>a</sup> Virus Infectivity Yield in LLC-Mk<sub>2</sub> Cells  
Maintained on Different Media

Inoculum (PFU/0.2 ml)	Media					
	M-199	M-199 +5% FCS	MEM	MEM +5% FCS	RPMI 1640	RPMI 1640 +5% FCS
1.5x10 <sup>3</sup> (TNTC) <sup>b</sup>	1 x 10 <sup>4</sup> c	2 x 10 <sup>3</sup>	7.2 x 10 <sup>4</sup>	7 x 10 <sup>4</sup>	3.8 x 10 <sup>6</sup>	5 x 10 <sup>5</sup>
1.5x10 <sup>2</sup> (45)	1.8 x 10 <sup>4</sup>	5 x 10 <sup>3</sup>	2 x 10 <sup>5</sup>	2 x 10 <sup>4</sup>	3.3 x 10 <sup>6</sup>	1 x 10 <sup>6</sup>
1.5x10 <sup>1</sup> (10)	2 x 10 <sup>3</sup>	2 x 10 <sup>3</sup>	2 x 10 <sup>4</sup>	2.9 x 10 <sup>4</sup>	4 x 10 <sup>4</sup>	1 x 10 <sup>4</sup>
1.5 (1.5)	7 x 10 <sup>1</sup>	1.8 x 10 <sup>1</sup>	6 x 10 <sup>2</sup>	8 x 10 <sup>2</sup>	1.6 x 10 <sup>4</sup>	7 x 10 <sup>4</sup>
0	0 <sup>d</sup>	0	0	0	0	0

a Dengue Type 3, 1337-74, Mk<sub>2</sub>-10

c PFU/0.2 ml of seeds harvested at day 7

b Actual count

d Virus not detected

TABLE 4. Dengue Type 4<sup>a</sup> Virus Infectivity Yield in LLC-Mk<sub>2</sub> Cells  
Maintained on Different Media

Inoculum (PFU/0.2 ml)	Media					
	M-199	M-199 +5% FCS	MEM	MEM +5% FCS	RPMI 1640	RPMI 1640 + 5% FCS
1 x 10 <sup>4</sup> (TNTC) <sup>b</sup>	1.6 x 10 <sup>4</sup> c	2.0 x 10 <sup>3</sup>	8.0 x 10 <sup>4</sup>	2.0 x 10 <sup>4</sup>	1.0 x 10 <sup>4</sup>	3.0 x 10 <sup>4</sup>
1 x 10 <sup>3</sup> (TNTC)	1.2 x 10 <sup>4</sup>	4.0 x 10 <sup>3</sup>	2.0 x 10 <sup>4</sup>	1.0 x 10 <sup>4</sup>	2.0 x 10 <sup>4</sup>	1.2 x 10 <sup>4</sup>
1 x 10 <sup>2</sup> (26)	1.0 x 10 <sup>4</sup>	1.5 x 10 <sup>1</sup>	7.0 x 10 <sup>2</sup>	1.0 x 10 <sup>4</sup>	1.0 x 10 <sup>4</sup>	5.1 x 10 <sup>4</sup>
1 x 10 <sup>1</sup> (3)	3.0 x 10 <sup>1</sup>	1.2 x 10 <sup>3</sup>	3.0 x 10 <sup>3</sup>	2.0 x 10 <sup>4</sup>	1.3 x 10 <sup>4</sup>	2.4 x 10 <sup>4</sup>
1 (1)	0 <sup>d</sup>	2.0 x 10 <sup>0</sup>	0	0	3.1 x 10 <sup>2</sup>	1.8 x 10 <sup>2</sup>

a Dengue Type 4, VN-H7-73, Mk<sub>2</sub>-10

b Actual count

c PFU/0.2 ml of seeds harvested at day 7

d Virus not detected

infection in the presence of RPMI 1640 medium, suggests that this medium might increase the chances of isolating dengue viruses from biological specimens. Therefore, RPMI 1640 medium will be employed in place of M-199 for our standard viral isolation procedures.

18. Glucose-6-Phosphate Dehydrogenase Deficiency and Dengue Hemorrhagic Fever

OBJECTIVE: To determine if Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency is related to the occurrence and/or course of dengue virus infection in man.

BACKGROUND: Despite a world wide distribution of dengue viruses, dengue hemorrhagic fever (DHF) and its severe manifestation, dengue shock syndrome (DSS) appear to be major problems only in Southeast Asia. It has been suggested that this may be due to the simultaneous circulation of two or more dengue virus serotypes in this part of the world. However, more than one serotype of dengue virus type is endemic in Africa and in the Caribbean and no DSS has yet been reported (Personal communication: COL Philip K. Russell, M.D.). Other factors which may be associated with the occurrence or the severity of dengue infections must also be studied. Glucose-6-Phosphate Dehydrogenase deficiency is a genetic enzyme deficiency which is an x-linked recessive trait. Although the enzyme deficiency has been observed in several tissues, it is recognized mainly in the erythrocyte, where it causes hemolytic anemia following exposure to certain drugs, food stuffs or infections. The disorder is fully expressed in hemizygous males and in the homozygous females. In the heterozygous female the expression varies and is dependent on the degree of expression of the normal gene.

In Thailand, G6PD deficiency has been reported in up to 14% of the Thai population (35). This condition has been found to occur more frequently in patients with bacterial infections such as typhoid fever or pneumococcal pneumonia than in non-infected control subjects. G6PD deficiency has been associated with viral as well as bacterial diseases. Morrow et al. (36) have pointed out a relationship between G6PD deficiency and the incidence as well as the severity of viral hepatitis. Since G6PD deficiency and DHF occur frequently in Thailand, a relationship was sought between the enzyme deficiency and the occurrence and/or severity of disease.

METHODS: Children hospitalized at the Bangkok Children's Hospital with signs and symptoms compatible with the diagnosis of DHF were

TABLE 1. Glucose 6 Phosphate Dehydrogenase Deficiency in Dengue Hemorrhagic Fever Patients and Controls

Subjects	No.	G6PD Deficiency*	
		No.	Percent
Patients	80	9	11.2
Controls	131	13	9.9

Chi square(1 d.f.) = 0.0053; p not significant

\* Includes all individuals homozygous, hemizygous or heterozygous for G6PD deficiency.

TABLE 2. Glucose 6 Phosphate Dehydrogenase Deficiency and the Severity of Dengue Infections

Dengue Patients	Total	Shock	
		No.	Percent
G6PD deficient*	9	7	77.7
G6PD normal	71	41	57.7
Total	80	48	60.0

Chi square (1 d.f.) = 0.6310; p not significant

\* Includes all individuals homozygous, hemizygous or heterozygous for G6PD deficiency.

studied for G6PD deficiency. G6PD was examined using the methemoglobin technique of Gall et al (37) which allowed for the determination of the patient's genotype. Patients were considered to have DHF if they met the laboratory criteria for diagnosis. A confirmed diagnosis was based on the isolation of the dengue virus and/or a four-fold or greater rise in antibody titer to dengue virus antigens. A presumptive diagnosis called for a fixed hemagglutination inhibition antibody titer  $\geq 1:640$  to two or more dengue virus antigens between the acute and convalescent sera. DHF presents a spectrum of severity. Grading of severity used established criteria (38).

Patients in this study were divided into two groups based upon the severity of illness. Those without circulatory failure and those with shock.

**RESULTS:** Eighty patients who met the laboratory diagnostic criteria for DHF were studied for G6PD deficiency. The frequency of G6PD deficiency in these patients was compared to a group of 131 controls collected in the well-baby clinic of the Bangkok Children's Hospital (Table 1).

There was a 11.2% prevalence of G6PD deficiency in patients with DHF while in the control group 9.9% were affected. There was no significant differences in the frequency of G6PD deficiency between the DHF groups and control groups. The frequency of homozygosity and hemizygosity for G6PD deficiency in the two populations was also not significantly different.

An association was sought between the presence of G6PD deficiency and the severity of dengue infection (Table 2). Of the 80 patients with DHF, nine were G6PD deficient. Of those nine with G6PD deficiency, seven developed shock, as compared to 41 out of 71 patients without G6PD deficiency. This difference was not significant.

The results show no significant relationship between G6PD deficiency and the occurrence of DHF. The relationship of G6PD deficiency and severity remains a question. Although there was no clear or significant relationship demonstrated this may have been due to the small number of G6PD deficient patients with shock that were studied. This project is now completed.

19. Biological and Immunological Studies of a Dengue 2 Candidate Virus Vaccine

**OBJECTIVE:** To investigate the immunological efficacy and mosquito infectivity of a candidate dengue 2 virus vaccine.

BACKGROUND: Dengue Hemorrhagic Fever (DHF) is a severe public health problem in Southeast Asia and represents a potential threat to military troops operating in areas where the dengue viruses are prevalent. For these reasons dengue virus strains are being sought for the preparation of vaccines. Over the past several years, a dengue 2 virus strain has been isolated under conditions suitable for vaccine development (39). The candidate vaccine virus (S1) is a small plaque variant isolated by plaque selection from dengue 2 PR-159, a strain obtained during the 1969 dengue 2 epidemic in Puerto Rico. Compared to the parent strain, (PR-159 PGMK-6), the small plaque variant (PR-159 S1-PGMK-19) was temperature sensitive and appeared to have decreased virulence in suckling mice. While the parent strain produced relatively consistent viremia in Indian Rhesus monkeys on days three to seven following inoculation, the S1 strain only occasionally produced viremia. When viremia did occur, it was as late as the tenth day following inoculation. Despite the inconsistency of viremia, the S1 strain appeared to be strongly immunogenic.

Following initial testing in the rhesus monkeys the S1 strain was further passed four times in fetal rhesus lung cells. A candidate vaccine (PR-159 (S1) Lot 1, PGMK-19 FRL-4) was prepared.

The purpose of this investigation was to study the clinical and immunological affect of this candidate vaccine strain on Indian Rhesus monkeys and to determine its infectivity for mosquitoes biting these monkeys.

METHODS: Two strains of dengue 2 virus were received from Dr. Walter E. Brandt, Department of Virus Diseases, Walter Reed Army Institute of Research. At the time of preparation the frozen parent strain (PR-159 PGMK-6) was reported to have a titer  $1.5 \times 10^5$  PFU/ml at  $35^\circ\text{C}$  and the lyophilized candidate vaccine strain (PR-159 (S1) PGMK-19 FRL-4, Lot 1) was reported to have a titer of  $1.9 \times 10^5$  PFU/ml at  $35^\circ\text{C}$ . The viruses were shipped on dry ice and held at  $-70^\circ\text{C}$ . The virus preparations were rapidly thawed and reconstituted just prior to use. Indian Rhesus monkeys (*Macaca mulatta*) were screened for hemagglutination inhibition (HI) antibody against dengue 1-4, Japanese encephalitis, wesselsbron and langat antigen and for neutralizing (N) antibody against dengue 2. Fifteen monkeys were selected which were negative for these antibodies. Monkeys received either the candidate vaccine or the parent strain. Each monkey was inoculated subcutaneously in the left arm with 0.5 ml of the appointed virus strain containing an estimated  $0.5 - 1 \times 10^5$  PFU. The monkeys were examined before inoculation and daily throughout the course of the experiment. General condition, appetite, temperature

organomegally, lymphadenopathy and presence or absence of petechia were noted. Blood was obtained prior to inoculation and on days 1 through 10, 15, 30 and 60 following inoculation. Blood was submitted for hemogram and platelet count.

Heparinized blood was separated and plasma and leukocytes were submitted for virus assay. Methods for separation of leukocytes are described elsewhere in this annual report (The Isolation of Dengue Viruses from Leukocytes). Virus isolations were attempted by the direct and delayed plaque assay technique. Serum was submitted for serology by HI tests and plaque reduction neutralizations tests (PRNT) against prototype dengue 104 antigens.

On days one through ten following inoculation; 30, 3-5 day old female mosquitoes provided by the Department of Medical Entomology were allowed to feed on each monkey. Engorged mosquitoes were separated, incubated for 14 days at 32°C, sacrificed by quick freezing and stored at -70°C. Ten mosquitoes were pooled, (one pool/monkey/day) sonicated in 1.0 ml of Roswell Park Memorial Institute (RPMI) 1640 media supplemented with glutamine, sodium bicarbonate and 10% fetal calf serum, and centrifuged at 1000 x g for 30 minutes. The supernatant was assayed for virus by direct and delayed plaque techniques. Six mosquitoes/day/monkey were decapitated; head squash preparations were examined for dengue antigen by a direct fluorescent antibody technique (FA).

Monkeys immunized with either the candidate vaccine or the parent dengue 2 strains were challenged with the candidate vaccine strain or with low passage Southeast Asian wild type strains of dengue 2 (BM-50-76 LLCM<sub>k</sub><sub>2</sub>-2) or dengue 3 (CH 1337-74 LLCM<sub>k</sub><sub>2</sub>-10). Dengue 2 (BM-50-76, LLC-M<sub>k</sub><sub>2</sub>-2) was isolated from an Aedes aegypti mosquito collected in the home of a DHF patient; dengue 3 (CH 1337-74, LLC-M<sub>k</sub><sub>2</sub>-10) was isolated from a DHF patient. The monkeys were handled and samples were collected for isolation and serology in a manner similar to that used in the initial infections.

RESULTS: Fifteen monkeys were inoculated with 0.5 ml of dengue 2 virus preparations. Ten of these received the candidate vaccine strain (PR-159 (S1) PGMK-19, FRL-4, Lot #1) and 5 received the parent strain (PR-159, PGMK-6) (Table 1). At the time of inoculation these virus preparations were titered at 37°C and found to contain  $6.7 \times 10^2$  and  $1.3 \times 10^5$  PFU/ml respectively. Examination of the 15 monkeys through out the course of the experiment showed no significant physical or hematological changes.

Using direct and delayed plaque assays at 37°C and 35°C no virus was recovered from the plasma of any of the ten monkeys who

received the candidate vaccine virus. Antibodies against dengue 2 virus had developed by 30 days following inoculation in six of these monkeys and HI antibody was found in three.

Viremia was documented in all five of the monkeys inoculated with the parent strain. Virus was isolated from the plasma of one monkey by the second day following inoculation and was found in all monkeys on days six and seven. The viremia persisted in most monkeys through day eight but had cleared by day nine. Dengue 2 virus was isolated from leukocytes taken from monkeys on days eight and nine, late in the course of infection. On two occasions virus was isolated from leukocytes but could not be detected in plasma. Both HI and N antibody to dengue 2 developed by the 15th day after immunization in all of the monkeys inoculated with the parent strain. The antibody titers at the time of measurement did not appear to be related to the time or duration of viremia.

Dengue virus was not detected by either isolation or FA in mosquitoes that fed on monkeys inoculated with the candidate vaccine strain. Infection was detected by direct and delayed plaque isolation and by FA only in mosquitoes fed on monkeys inoculated with the parent virus. Virus was intermittently isolated from mosquitoes that fed on these monkeys on day three through six following inoculations. Isolations could be obtained from mosquitoes at times when no virus could be detected in the plasma, and virus was usually isolated from mosquitoes before or early in the stage of detectable viremia.

Challenge experiments were carried out 126 days following initial inoculation (Table 2). The monkeys which had received the candidate vaccine strain were divided into groups based upon the N antibody titer to dengue 2 antigen at 30 days. Three pairs were established each contained one monkey with an N antibody titer of  $\leq$  1:20 and one with a titer of  $\geq$  1:20. These pairs were inoculated with the candidate vaccine strain, and low passage Southeast Asian wild type strains of dengue 2 (BM-50-76, LLC-Mk<sub>2</sub>-2) and dengue 3 (CH 1337-74, LLC-Mk<sub>2</sub>-10) respectively. Of the remaining four monkeys who did not develop N antibody following inoculation with the candidate vaccine strain, one was inoculated with the candidate vaccine strain, two were challenged with the wild type dengue 2 strain and one received the wild type dengue 3 virus.

Plasma collected from all monkeys just prior to challenge were retested for HI and N antibodies. Of the monkeys which had received the candidate vaccine strain, only one monkey had N

Table 1. Viremia and Antibody Responses Following Primary Inoculation of Indian Rhesus Monkeys with Dengue Viruses

Monkey Number	Primary Inoculum	Viremia Day							Dengue 2 Antibody Day						
		1	2	3	4	5	6	7	8	9	10	0	10	15	30
E228	Vaccine (D2) <sup>a</sup>	-	-	-	-	-	-	-	-	-	-	0/0c	0/	0/10	20/20
E231	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	0/	0/5	0/5
E290	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	0/	0/0	0/0
E293	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	0/	0/0	0/0
E294	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	0/	0/0	0/0
E297	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	0/	0/65	0/80
E298	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	0/	0/0	0/0
E299	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	0/	0/0	80/140
E301	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	0/	0/20	40/53
F15	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	0/	0/10	0/10
F17	Parent (D2) <sup>b</sup>	-	-	+	+	+	+	+	+	+	△	-	0/0	0/	80/130
F66	Parent (D2)	-	-	-	-	+	+	+	+	+	△	-	-	0/0	160/220
F67	Parent (D2)	-	-	-	-	-	-	+	+	+	+	-	-	0/0	160/680
F70	Parent (D2)	-	-	-	-	-	-	□	+	+	△	-	-	40/	320/1000
F74	Parent (D2)	-	+	□	+	+	+	+	+	+	+	-	-	20/	320/220
												-	-	10/	320/340
												-	-		320/1000

a Dengue 2 Candidate Vaccine (PR-159, (S1) Lot 1, June 76)

b Dengue 2 Parent Strain (PR-159, PGMK-6)

c Reciprocal Hemagglutination Inhibition/Neutralization Titers, 0 = < 1:10

+ = Isolation of Dengue 2 from Plasma  
 □ = Isolation of Dengue 2 from Mosquitoes  
 Δ = Isolation of Dengue 2 from Leukocytes  
 - = No isolation

Table 2. Viremia and Antibody Responses Following Secondary Inoculation of Indian Rhesus Monkeys with Dengue Viruses

Monkey Number	Secondary Inoculum	Viremia Day										Dengue 2 Antibody Day		
		1	2	3	4	5	6	7	8	9	10	126	141	156
E228	Vaccine (D2) <sup>a</sup>	-	-	-	-	-	-	-	-	-	-	0/0 <sup>d</sup>	160/200	80/250
E231	BM50-76 (D2) <sup>b</sup>	-	-	-	+	▲	▲	-	-	-	-	0/0	1280/ ≥640	640/ ≥640
E290	BM50-76 (D2)	-	-	▲	▲	-	-	-	-	-	-	0/0	5120/ ≥640	640/ ≥640
E293	BM50-76 (D2)	-	-	+	△	-	-	-	-	-	-	0/0	80/90	80/170
E297	CH1337-74 (D3) <sup>c</sup>	-	-	+	+	-	-	-	-	-	-	0/0	640/250	320/320
E298	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	160/300	80/160
E299	BM50-76 (D2)	-	-	-	-	-	-	-	-	-	-	80/100	320/ ≥640	160/ ≥640
E301	Vaccine (D2)	-	-	-	-	-	-	-	-	-	-	0/0	80/180	40/160
F15	CH1337-74 (D3)	-	-	▲	+	-	-	-	-	-	-	0/0	320/320	160/550
F17	CH1337-74 (D3)	-	-	+	+	+	-	-	-	-	-	160/300	1280/340	1280/190
F66	CH1337-74 (D3)	-	-	-	-	-	-	-	-	-	-	160/ ≥640	5120/ ≥640	2560/ ≥640
F67	BM50-76 (D2)	-	-	-	-	-	-	-	-	-	-	320/ ≥640	1280/ ≥640	1280/ ≥640
F70	BM50-76 (D2)	-	-	-	-	-	-	-	-	-	-	640/ ≥640	\$120/ ≥640	256/ ≥640

<sup>a</sup> Dengue 2 Candidate Vaccine (PR-159, (S1) Lot 1, June 76)

<sup>b</sup> BM-50-76, LLC-Mk2, Southeast Asian Dengue 2

<sup>c</sup> CH 1337-74, LLC-Mk2, Southeast Asian Dengue 3

<sup>d</sup> Reciprocal Hemagglutination Neutralization Titters 0=< 1:10

+ = Isolation of Dengue 2 from Plasma  
 □ = Isolation of Dengue 2 from Mosquitoes  
 △ = Isolation of Dengue 2 from Leukocytes  
 - = No isolation

and/or HI antibody at this time, suggesting that the antibody detected at 30 days might have been of the IgM class. All the monkeys which had received the parent strain retained both N and HI antibodies.

The titers of inoculum at the time of injection, determined by plaque assay at 35°C were  $5 \times 10^3$  PFU/ml for the candidate vaccine strain,  $1.1 \times 10^5$  PFU/ml for the wild type dengue 2 strain (BM-50-76) and  $4.6 \times 10^5$  PFU/ml for the wild type dengue 3 strain (CH-1337-74). In the monkeys reimmunized with the candidate vaccine strain, there was no detectable viremia; none the less all of these monkeys developed appreciable titers of dengue 2 HI and N antibody by day 141, 15 days after reimmunization. These antibodies persisted at essentially the same titer for at least one month after immunization.

Of the four vaccinated monkeys challenged with the dengue 2 strain (BM-50-76), three developed viremia and leukocyte infections on the fourth through the sixth day after challenge. The fourth which developed no demonstrable infection was the one monkey which had appreciable amounts of dengue 2 HI and N antibody just prior to challenge. Challenge of two monkeys that received the parent strain of dengue 2 virus (PR-159, PGMK-6), with wild type dengue 2 (BM-50-76) resulted in HI titers rises but no demonstrable viremia. N antibody was not tested at a level necessary to detect a titer rise, it was already  $\geq 1:640$  at the time of challenge.

Four monkeys were challenged with the dengue 3 wild type virus (CH-1337-74). Two of these, having previously received candidate dengue 2 vaccine virus, developed dengue 3 viremia and cross reactive HI and N antibodies. The other two were initially inoculated with the dengue 2 parent strain (PR-159, PGMK-6). Of these, only one developed a viremia. This occurred at approximately the same time as the viremia occurred in the previous immunization with the dengue 2 parent strain. Both of these monkeys developed cross reactive HI and N antibodies.

This work is now completed and will be prepared for publication.

#### 20. Dengue Virus Isolation from Patients and Mosquitoes

OBJECTIVE: To provide dengue viruses, isolated from patients and Aedes aegypti mosquitoes, for the study of dengue virus markers.

BACKGROUND: Previous studies (40) have demonstrated that the homes of dengue patients provided the most abundant sites of dengue infected A. aegypti mosquitoes. Mosquitoes collected from patient's homes were used to isolate virus for dengue virulence studies. This collection also expanded our knowledge of the relative densities of dengue vectors, both infectious and uninfectious, in the vicinity of dengue patients.

METHODS: Indoor daylight collections of adult mosquitoes, using the pyrethrin spray knockdown techniques, were made in the homes of clinical patients who were admitted to the Bangkok Children's Hospital dengue hemorrhagic ward. Mosquitoes from each house were then identified and frozen in suitable size pools for virus isolations. A direct and delayed plaque technique was employed for virus isolation and viruses were identified by a plaque reduction neutralization test.

RESULTS: Forty-nine patient homes were visited by an entomology team between the 20th of June and 1 December 1976. Serological studies of patients residing in these houses revealed that only 45 of the 49 patients had dengue infections. In the 45 houses of these laboratory diagnosed dengue patients a total number of 281 A. aetypti were collected (average mosquitoes per house, 6.2, range 0-50 mosquitoes). There were 161 engorged mosquitoes, (average per house 3.6, range 0-20) and 120 unengorged mosquitoes (average per house 7.2, range 0-29). Of the 45 houses studied, ten provided engorged mosquitoes and 28 harbored both engorged and unengorged mosquitoes. Five houses revealed only unengorged mosquitoes, and two houses had no mosquitoes at all. For each house engorged and unengorged pools of mosquitoes were submitted for isolation. From the mosquitoes collected from the 43 houses of the DHF patients there was one isolation made. It was made from an unfed mosquito pool collected one day after a resident of the house was admitted to the hospital. Besides the isolation of dengue 2 from the mosquito (BM-50-76) dengue two was also isolated from the patient (D76-015). This dengue 2 mosquito isolate has been used as a Southeast Asian dengue 2 wild type strain in vaccine studies.

Further collections of this type are probably warranted if done on a large scale in association with an intensive study of dengue virulence markers or mosquito ecology.

21. Epidemiology of Hepatitis B in a Well Defined Rural  
Thai Population

OBJECTIVE: To determine the prevalence of hepatitis B surface antigen ( $\text{HB}_S\text{Ag}$ ) and antibody to hepatitis B surface antigen (anti- $\text{HB}_S$ ) in a well defined rural Thai village.

BACKGROUND: A study on a well defined urban Thai population (41) has shown an average prevalence of  $\text{HB}_S\text{Ag}$  of 8.2% (determined by radioimmune assay) and anti- $\text{HB}_S$  of 46.1% (determined by passive hemagglutination). In this population of 697 people aged from one year to 75 years, the prevalence of  $\text{HB}_S\text{Ag}$  was relatively stable throughout all age groups. For anti- $\text{HB}_S$  on the other hand, the prevalence rose from 15.4% in the 1-4 age group to a plateau level of 50 to 65% after the age of 20. A stable rural Thai population was sought to determine the prevalence of  $\text{HB}_S\text{Ag}$  and anti- $\text{HB}_S$  for comparison with that found in the Bangkok population.

METHODS: The village of Tablan was selected for study. This village was located in Prachinburi province in the Bu Phram valley. Its population had been included in malaria drug prophylaxis studies for two years. Sera from a portion of the population had been collected for malaria studies at approximately yearly intervals in 1974 and 1975. A census of the village conducted in early 1976 showed the total population to consist of 1,014 people, 503 males and 511 females. Sera were assayed for  $\text{HB}_S\text{Ag}$  and anti- $\text{HB}_S$  serology by radioimmune assay (ASURIA II and AUSAB supplied by Abbott Laboratories, North Chicago, Ill.).

RESULTS: Blood was obtained from 73% of the village population in late 1976 (Table 1). The prevalence of  $\text{HB}_S\text{Ag}$  carriers in the total population tested was 7.2% (Table 2). There was no  $\text{HB}_S\text{Ag}$  detected among 70 tested children under the age of four. After that, the prevalence in males was significantly higher than for females in all but one age group (10-14 years of age). There was no significant difference in prevalence of  $\text{HB}_S\text{Ag}$  by age group except in the children under four years of age.

Anti- $\text{HB}_S\text{Ag}$  was found in 31.25% of the population (Table 3). As in the Bangkok population, the prevalence of anti- $\text{HB}_S$  rose from 11.4% in the one to four age group and reached a plateau level of about 50% by age 20. The time of rise was essentially the same in both populations but the rural population did not reach the levels seen in the urban population. This difference may be greater than it appears because of the increased sensitivity of

TABLE 1  
Population of Ban Tablan that was Selected  
for Hepatitis B Virus Investigation

Age (year)	Population in Village	MALE		FEMALE		TOTAL			
		Tested No.	%	Population in Village	No.	Tested %	Population in Village	No.	%
0-4	86	33	38.37	94	37	39.36	180	70	38.89
5-9	75	59	78.66	79	69	87.34	154	128	88.89
10-14	77	64	83.12	76	66	86.84	153	130	84.97
15-19	56	36	64.28	45	34	75.56	101	70	69.31
20-29	69	54	78.26	84	67	79.76	153	121	79.08
30-39	50	40	80.0	53	45	84.9	103	85	82.52
40-49	47	36	76.59	35	27	77.14	82	63	76.83
50-59	28	20	71.43	30	26	86.67	58	46	79.31
60 <sup>+</sup>	15	11	73.33	15	12	80.0	30	23	76.67
Total	503	353	70.3	511	383	75.0	1,014	739	72.9

TABLE 2  
Prevalence of HB<sub>S</sub> Ag in Residents of Ban Tablan

Age (Year)	MALE			FEMALE			TOTAL		
	No. Tested	Positive		No. Tested	Positive		No. Tested	Positive	
		No.	%		No.	%		No.	%
0-4	33	0	0	37	0	0	70	0	0
5-9	59	7	11.86	69	4	5.79	128	11	8.59
10-14	64	3	4.68	66	7	10.6	130	10	7.69
15-19	36	4	11.11	34	3	8.82	70	7	10.0
20-29	54	8	14.8	67	3	4.48	121	11	9.09
30-39	40	5	12.5	45	0	0	85	5	5.88
40-49	36	3	8.3	27	0	0	63	3	4.76
50-59	20	2	10.0	26	2	7.7	46	4	8.69
60 <sup>+</sup>	11	2	18.2	12	0	0	23	2	8.69
Total	353'	34	9.63	383	19	4.96	736	53	7.2

TABLE 3  
Prevalence of Anti-HB<sub>S</sub> in Residents of Ban Tablan

Age (Year)	MALE			FEMALE			TOTAL		
	No. Tested	Positive		No. Tested	Positive		No. Tested	Positive	
		No.	%		No.	%		No.	%
0-4	33	2	6.06	37	6	16.2	70	8	11.42
5-9	59	11	18.64	69	15	21.74	128	26	20.3
10-14	64	24	37.5	66	23	34.84	130	47	36.15
15-19	36	19	52.78	34	9	26.47	70	28	40
20-29	54	27	50.0	67	26	38.80	121	53	43.8
30-39	40	19	47.5	45	22	48.89	85	41	48.2
40-49	36	22	61.1	27	12	44.4	63	34	53.97
50-59	20	12	60.0	26	14	53.8	46	26	56.52
60 <sup>+</sup>	11	10	90.9	12	10	83.3	23	20	86.96
Total	353	146	41.36	383	137	35.8	736	230	31.25

the serological test used for the rural Thai population. Males after the age of 15 had a significantly higher prevalence of antibody than did the females of equivalent age. Unlike the Bangkok population, however, this difference in prevalence was observed for all of the older age groups.

Further analysis of the prevalence data with the addition of family prevalence and longitudinal follow-up are required to complete this study.

22. A Longitudinal Study of Hepatitis B Virus Infection in a School Population

OBJECTIVE: To conduct a longitudinal study of hepatitis B virus infections in a lower socioeconomic school population.

BACKGROUND: Studies of a well-defined population of lower socioeconomic Bangkok residents (Huay Khwang) have shown that 8.2% of the population have hepatitis B surface antigen ( $\text{HB}_S\text{Ag}$ ) in their blood (42). Children in the 1-4 year age group had a 4.5% prevalence rate. There was no significant difference in antigen prevalence between age groups. Antibody to  $\text{HB}_S\text{Ag}$  (anti- $\text{HB}_S$ ) on the other hand, was noted to increase rapidly from 15.4% to 48.9% between the ages of one and 19 years. These data suggested that hepatitis B virus (HBV) is widely disseminated in the population studied. Data from Huay Khwang also suggested that the risk of acquiring hepatitis B antigen, but not antibody, was associated with the family unit.

Two groups of mothers and their offspring have been studied. The  $\text{HB}_S\text{Ag}$  carrier rate in these mothers was not statistically different from that seen in women of child-bearing age in Huay Khwang. Follow-up of the mothers and offspring have shown that acquisition of HBV infection by infants in the first year occurred almost exclusively in families in which the mother was a  $\text{HB}_S\text{Ag}$  carrier (43). Fifteen of 31 (48%) infants of antigenemic mothers became infected by the age of 6 months and, of these nine (60%) became chronic carriers. Antigen and antibody acquisition data suggested the possibility of an extra-familial sources of infection after the first year of life. This report consists of preliminary findings of a longitudinal study designed to determine the incidence of HBV infection and the change in the prevalence of  $\text{HB}_S\text{Ag}$  carriers in a school population. The same school group was also used for a longitudinal study of dengue virus infection.

METHODS: Volunteer students attending the Pibulprachansan school in the Dindaeng area of Bangkok were studied. A consent

TABLE 1. The Prevalence of HB<sub>S</sub>Ag Markers in Pibulprachasan School Students

Age	Male			Female			Total	
	No. Tested	HB <sub>S</sub> Ag No. (+) (%)	No. Tested	HB <sub>S</sub> Ag No. (+) (%)	No. Tested	HB <sub>S</sub> Ag(+) No. (%)		
4	15	2 (13.3)	33	1 (3.0)	48	3 (6.3)		
5	30	2 (6.7)	33	1 (3.0)	63	3 (4.8)		
6	57	2 (3.5)	35	1 (3.0)	90	3 (3.3)		
7	85	7 (8.2)	103	5 (4.9)	188	12 (6.4)		
8	115	7 (6.1)	88	6 (6.8)	203	13 (6.4)		
9	116	11 (9.5)	110	5 (4.5)	226	16 (7.1)		
10	123	11 (8.9)	127	12 (9.5)	250	23 (9.2)		
11	108	10 (9.3)	107	3 (2.8)	215	13 (6.0)		
12	115	6 (5.2)	131	4 (3.1)	246	10 (4.1)		
13	111	11 (9.9)	168	9 (5.4)	279	20 (7.2)		
14	42	3 (4.1)	79	3 (3.8)	121	6 (5.0)		
15	8	1 (5.6)	28	3 (10.7)	36	4 (1.1)		
Total	935	71 (7.8)	1042	53 (5.1)	1977	126 (6.4)		

TABLE 2. Distribution of HB<sub>S</sub>Ag Subtypes in Student HB<sub>S</sub>Ag Carriers  
in the Pibulprachansan School

Sex	Number Tested	Subtypes		
		adr	adw	ad?
Female	50	39 (78.0) <sup>a</sup>	9 (18.0)	2(4.0)
Male	68	61 (89.7)	6 (8.8)	1(1.5)
Total	118	100 (84.7)	15 (12.7)	3(2.6)

a Number parenthesis = Percent of number tested

form, signed by a parent or guardian and a family history was obtained from each student. Volunteers were bled in May of 1977, the first month of school. Blood will be obtained again from each student in December and in April of the same academic year.

Blood was submitted for initial screening for HBV markers by IEOP. Final testing was accomplished by radioimmune assay for HB<sub>S</sub>Ag (AUSRIA II) and anti-HB<sub>S</sub> (AUSAB). (Both radioimmune assay kits were provided by the Abbott Laboratories, North Chicago, Ill.) Each antigen detected was further tested for subtype by immunodiffusion.

RESULTS: Sera of 1977 students including 935 males and 1042 females were screened for HBV markers (Table 1). The age specific point prevalence of HB<sub>S</sub>Ag in the whole group was 6.4% with no significant differences by age group. Although the differences were not significant, there was a higher prevalence of HB<sub>S</sub>Ag carriers among males than females. Subtype determinations of the HB<sub>S</sub>Ag showed 80.9% adr and 11.5% adw, (Table 2) figures which are compatible with those previously found in Bangkok populations (44). This is an ongoing study. Data collected on the next two bleeds should allow for calculation of incidence of HBV infection and point to modes of transmission that should be investigated.

### 23. The Frequencies of Hepatitis B Antigen Subtypes in Various Parts of Thailand

OBJECTIVE: To determine the prevalence of hepatitis B surface antigen (HB<sub>S</sub>Ag) subtypes in different regions of Thailand.

BACKGROUND: This is the conclusion of an investigation that was reported previously (45). The surface of the hepatitis B virus (HBV) contains at least five different antigenic determinants; the common determinant a, and two pairs of usually mutually exclusive determinants d/y and w/r. This allows for four different subtypes; two ad subtypes, adw and adr, and two ay subtypes, ayw and ayr.

In Southeast Asia, the HB<sub>S</sub>Ag subtypes detected are almost universally ad. Approximately 80% of the ad carriers had a third r determinant, while the remaining 20% carried w determinants (46). In other countries in Asia increases in the prevalences of the r determinant are found with movement from north to south (47,48). These observations suggested that subtype prevalence might also relate to geographic areas in Thailand.

TABLE 1

The Relative Frequency of HB<sub>S</sub>Ag Subtypes in Blood Donors  
of Different Geographical Areas of Thailand

Region	HB <sub>S</sub> Ag Positive Sera	HB <sub>S</sub> Ag Subtypes		
		adr	adw	ad
	No.	(%)	No.	(%)
North <sup>a</sup>	39	36 (94)	3 (6)	0 (0)
East <sup>b</sup>	91	76 (84)	12 (13)	3 (3)
Central <sup>c</sup>	252	191 (76)	43 (17)	18 (7)
South <sup>d</sup>	45	38 (84)	4 (9)	3 (7)
Total	444	355 (80)	64 (14)	25 (6)

a. North: Chiang Mai

b. East: Cholburi, Trat, Chantaburi

c. Central: (Excluding Bangkok), Prathom Thani, Nontaburi  
Ayudhaya, Lopburi, Pichit, Rajburi, Nakorn  
Pathom, Samut Prakarn, Phetchaburi,  
Kanjanaburi, Samut Sakorn, Samut Songkram,  
Prachuab Kirikan

d. South: Songkla, Hat Yai

METHODS: This study was done in collaboration with the Thai Red Cross Center, Bangkok, The Chiangmai University Blood Bank and the Prince of Songkla University Blood Bank. Sera of blood donors were tested for the presence of HB<sub>S</sub>Ag by immunoelectroosmophoresis (IEOP). Antigen subtypes were determined by immuno-diffusion (ID) using specific rabbit antisera.

RESULTS: The prevalence of HB<sub>S</sub>Ag subtypes in populations of different geographic regions in Thailand are presented in Table 1. The adr subtype was found in 80% of the total positive sera. The adw subtype was observed in 14%. There was a statistically significant difference in the prevalence of adr between the North of Thailand (Chiangmai) and the central portion of the country. Other portions of the country had slightly higher prevalences than the central area. However, these differences were not significant.

Thus, there appeared to be a small but significant increase in the prevalence of adr as one moved north in Thailand; this gradient was small compared to those reported in Japan and China.

#### 24. The Prevalence of e-Antigen in Acute and Chronic Hepatitis in Thailand

OBJECTIVE: To determine the prevalence of e-antigen (HB<sub>e</sub>Ag) in patients with acute and chronic liver disease and to compare this with that found in asymptomatic hepatitis B surface antigen (HB<sub>S</sub>Ag) carriers.

BACKGROUND: Three antigen-antibody systems have been associated with hepatitis B virus (HBV) infection: HB<sub>S</sub>Ag and its antibody (Anti-HB), hepatitis B core antigen (HB<sub>C</sub>Ag) and its antibody (anti-HB<sub>C</sub>) and e antigen (HB<sub>e</sub>Ag) and its antibody (anti-HB<sub>e</sub>). HB<sub>S</sub>Ag and HB<sub>C</sub>Ag are both particulate viral antigens while HB<sub>e</sub>Ag is a soluble, probably non-viral, antigen which appears to be associated with the presence of Dane particles and DNA polymerase activity (49,50). The presence of HB<sub>e</sub>Ag has been reported by several investigators to be useful as a prognostic indicator (51). It was often found in patients with chronic aggressive hepatitis (CAH) but was unusual in chronic persistent hepatitis (CPH); anti-HB<sub>e</sub> has been reported more often in CPH and asymptomatic HB<sub>S</sub>Ag carriers than in patients with active disease (52). The prevalence of HB<sub>S</sub>Ag carriers (8-10%) and the incidence of cryptogenic cirrhosis are both high in Thailand.

This study was undertaken to investigate whether HB<sub>e</sub>Ag may be used to indicate severe or progressive liver disease.

TABLE 1. The Prevalence of HB<sub>S</sub>Ag, HB<sub>e</sub>Ag and anti-HB<sub>e</sub> in People with Hepatitis and a Comparative Group of Blood Donors

Diagnosis	Number Tested	HB <sub>S</sub> Ag (+)		HB <sub>e</sub> Ag (+)		Anti-HB <sub>e</sub> (+)	
		No.	%	No.	%	No.	%
Hepatitis Patients							
CAH <sup>a</sup>	16	10	62.5	1	6.3	1	6.3
AH <sup>b</sup>	30	14	46.6	0	0	3	10
Blood Donors	585	47	8.0	4	0.7	14	2.4

a Chronic active hepatitis diagnosed by liver biopsy

b Acute viral hepatitis diagnosed clinically

TABLE 2. The Prevalence of HB<sub>e</sub>Ag and Anti-HB<sub>e</sub> in HB<sub>s</sub>Ag Carriers

Diagnosis	HB <sub>s</sub> Ag (+)	HB <sub>e</sub> Ag (+)		Anti-HB <sub>e</sub> (+)	
		No.	%	No.	%
Hepatitis Patients CAH <sup>a</sup>	10	1	0	1	10.4
VH <sup>b</sup>	14	0	0	3	21.4
Blood Donors	47	4	8.5	14	29.7

a Chronic active hepatitis diagnosis by liver biopsy

b Acute viral hepatitis diagnosed clinically

METHODS: Blood was collected from patients seen in the hepatitis clinic at the Siriraj Hospital. A comparative group of asymptomatic carriers were obtained from the Blood Bank of the Royal Thai Army Hospital. Serum was tested for HB<sub>S</sub>Ag by immunoelectroosmophoresis (IEOP) and for HB<sub>e</sub>Ag and anti-HB<sub>e</sub> by IEOP or Immunodiffusion (ID). These methods have been described in previous Annual Reports (49).

RESULTS: Serum was obtained from 16 patients with biopsy proved CAH and 34 patients with clinically diagnosed acute viral hepatitis. The results of serological tests on these patients were compared with those on 585 blood donors. As expected, HB<sub>S</sub>Ag was prevalent in the patients with chronic and active hepatitis than in the blood donors (Table 1). HB<sub>e</sub>Ag, however, was found with low and equal frequency among the CAH patients and blood donors (Table 2). Essentially similar findings were seen with anti-HB<sub>e</sub> (Table 2). It is concluded that in our hands e antigen cannot be used as an indicator of severe liver disease. Determination of HB<sub>S</sub>Ag, HB<sub>e</sub>Ag and anti-HB<sub>e</sub> will be completed on a larger group of patients and an age and sex matched control group. Following this, final analysis of the prevalence of e antigen in chronic hepatitis will be completed.

25. The Epidemiology of Viral Hepatitis in Americans in Southeast Asia

OBJECTIVE: To study the epidemiology of hepatitis in American military personnel exposed to populations with endemic hepatitis and a high prevalence of hepatitis B surface antigen (HB<sub>S</sub>Ag) carriers.

BACKGROUND: In order to make decisions about disease control, the epidemiology of the disease must be understood. Viral hepatitis has long been a major military problem. In the Vietnam conflict, it was the second most frequent cause for the loss of man hours. Until recently, only historical evidence was available to document infection with agents causing hepatitis. In the past decade however, serological tests have been devised which identify both hepatitis A and hepatitis B virus infection. This report is the conclusion of a study reported in previous annual reports (52). Here we report data on the subtype specificities of antibody to hepatitis B surface antigen (anti-HB<sub>S</sub>) in those people who developed antibody, a partial analysis of behavioral patterns associated with the development of hepatitis B and data on the incidence and prevalence of hepatitis A infections.

METHODS: Subjects were drawn from servicemen aged 18-27 years in enlisted grades E-1 to E-5. These men were assigned to either the United States Army Support Group, Thailand or the United States Air Force 635th Combat Support Group. Shortly after arrival in Thailand a questionnaire was administered to volunteers to determine personal demographic and medical information. During the ensuing year these men were interviewed 3 times at approximately 4 month intervals regarding social, behavior and medical problems. Serum samples were collected at the time of each interview and submitted for serological tests for evidence of hepatitis A and B virus infections. Data on the prevalence and incidence of HBV infections were presented previously (52).

The radioimmune assay inhibition test used to identify the subtype of anti-HB<sub>S</sub> was similar to that published by Hoofnagle et al (53). This utilized the commercially prepared solid phase radioimmune assay for anti-HB<sub>S</sub> (AUSAB, Abbott Laboratories, North Chicago, Ill.). Subtype specificity of anti-HB<sub>S</sub> was identified by incubation of aliquots (0.1 ml) of serum containing anti-HB<sub>S</sub> with equal volumes (0.1 ml) of serum containing known HB<sub>S</sub>Ag subtypes, adr, adw and ayw. A control sample incubated with normal human serum was included. Following 2 hours incubation at room temperature, test beads (coated with HB<sub>S</sub>Ag) supplied in the commercial kit, were added and the test proceeded according to the instructions of the manufacturer. The counts per minute resulting from the mixture of the serum with each subtype and that resulting from the mixture with normal human serum were compared. Subtype specificity of antibody could be identified if the counts per minute were reduced by the addition of any HB<sub>S</sub>Ag subtype to less than 50% of that at the normal human serum control. The serum was considered to contain antibody to the shared subtype determinants if 2 or more HB<sub>S</sub>Ag subtypes reduced the counts per minute by approximately equal degree.

Hepatitis A antibody was detected by the specific immune adherence technique described by Miller et al (54). These tests were kindly performed for us by Drs. P.J. Provost and M.R. Hilleman of the Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, in West Point, Penna.

RESULTS: Of 18 people who acquired anti-HB<sub>S</sub> during a year follow up period, 16 yielded sufficient serum for testing. In 14 of these men we were able to detect antibody to 2 subtype determinants and in 8 we were able to identify antibody to 3 subtype determinants. Ten persons had antibody directed against the ad determinants. In 6 of these, antibody to the third determinant could be identified, one against adw and five against adr. Four of the 10

Table 1. Significance Level<sup>1</sup> of Variables Associated with Hepatitis B Virus Infections

Variable	Probability <sup>2</sup>
1. Race (being black)	a
2. Use of Thai language	a
3. Weeks off base first three months	b
4. Ever living off base second time	a
5. Ever living off base in tour	c
6. Weeks with hired wife first three months	b
7. Ever having hired wife second three months	c
8. Ever having hired wife in tour	d
9. Total of hired wives in tour	b
10. Total cases VD (or NGU) in tour	c
11. Total sex contacts first three months	a
12. Total sex contacts second three months	a
13. Total sex contacts third three months	d
14. Total sex contacts in tour	a
15. Marijuana use second three months	b
16. Total marijuana use in tour	a
17. Having friends of hired wife using marijuana	a

1. In each case, individuals with hepatitis B virus infections had significantly more of the behavior in question.

2. a)  $p \leq .05$  b)  $p \leq .02$  c)  $p \leq .01$  d)  $p \leq .001$

people who developed antibody against ad had it by the end of the first 3 months of their stay in Thailand. The remaining 6 acquired it later.

There were 4 persons who developed antibodies to ay subtypes. Three were 4 persons who developed antibodies to ay subtypes. Three of these persons developed them during their first 3 months in Thailand. These individuals probably did not acquire their hepatitis B infections from contact with the Thai population, as Thais carry almost exclusively ad subtypes.

Computer analyses of behavioral data compiled at the time of interview have identified 4 major areas of activity which correlate with development of hepatitis B infection during the course of one year in Thailand.

Individuals acquiring HBV infections had significantly more social and sexual contact with the Thais than did those who were not infected. The infected individuals usually lived in the Thai community and were more likely to be marijuana smokers. (Table 1 shows those variables which were significantly associated with the acquisition of HBV).

Serum collected on arrival and after one year in Thailand from 176 people were tested for antibody to hepatitis A. Of these, 35 (19.8%) had antibody on arrival in Thailand. Of a number of variables tested, including educational levels, size of home towns and age, only race (that is, being black) was significantly associated with prior hepatitis A infection.

There were 5 people who developed antibodies to hepatitis A while in Thailand, 4 of these had subclinical infections. One man was hospitalized with icteric hepatitis. This man was one of 6 who had clinical hepatitis; the other 5 were diagnosed as hepatitis B (52). Because of the few cases of hepatitis A, no behavioral correlates of hepatitis A acquisition could be defined.

This completes the laboratory work and the computer analysis on this project. The data will be prepared for publication.

26. The Prevalence of Hepatitis B Virus Markers in Dengue Hemorrhagic Fever Patients

OBJECTIVE: To study the prevalence of hepatitis B surface antigen ( $\text{HB}_s\text{Ag}$ ) carriers among patients with dengue hemorrhagic fever.

TABLE 1. Prevalence of HB<sub>S</sub>Ag in Patients with Dengue Infection

Age (Year)	Male		Female		Total	
	No. Tested	HB <sub>S</sub> Ag No. (+) (%)	No. Tested	HB <sub>S</sub> Ag No. (+) (%)	No. Tested	HB <sub>S</sub> Ag No. (+) (%)
1-4	7	0 (0)	3	0 (0)	10	0 (0)
5-9	18	1 (5.5)	19	2 (10.5)	37	3 (8.1)
10-14	14	1 (7.1)	9	0 (0)	23	1 (4.3)
Total	39	2 (5.1)	31	2 (6.6)	70	4 (5.7)

BACKGROUND: Hepatitis B virus can cause a chronic viral infection. The question has been asked whether the presence of a chronic viral infection interferes with infections by other viral agents. As studies were ongoing on dengue infected children, this question was examined by screening hospitalized dengue patients for HB<sub>S</sub>Ag assays.

MATERIAL & METHODS: Patients with serologically confirmed dengue infections were selected for the study. Convalescent sera were tested for HBsAg by radioimmune assays. (AUSRIA II, Abbott Laboratory, North Chicago, Ill.)

RESULTS: There were 70 patients selected between age one and 14 years of age (Table 1). The prevalence of HB<sub>S</sub>Ag in this group was 5.7%. This was not significantly different from the mean prevalence for HBsAg found in the urban Thai population (55). There was no apparent relationship between hepatitis B virus carriers and hospitalized dengue patients indicating that interference between these two viruses does not occur. Further investigations are not considered necessary.

#### 27. The Relationship between Glucose-6-Phosphate Dehydrogenase Deficiency and Hepatitis B Virus Infection

OBJECTIVE: To determine the role, if any, of glucose-6-phosphate dehydrogenase (G6PD) deficiency in the susceptibility to hepatitis B virus infection.

BACKGROUND: G6PD deficiency has been reported by Morrow et al (56) to be associated with both the incidence and the severity of viral hepatitis in West Africa. We sought such an association between G6PD deficiency and evidence of hepatitis B virus infection in Thailand.

METHODS: Professional blood donors at the Pramongkhutkao Hospital blood bank were selected for the study. Serum of each individual was assayed for hepatitis B virus surface antigen (HB<sub>S</sub>Ag) and antibody to HB<sub>S</sub>Ag (anti-HB<sub>S</sub>) by immunoelectroosmophoresis (IEOP). The level of G6PD was determined by the method of Gall et al (57).

RESULTS: Of 163 human sera, 18 (11%) contained HB<sub>S</sub>Ag and 45 (27.6%) contained anti-HB<sub>S</sub>. These findings are similar to previous studies on Thai blood donors (58). G6PD deficiency was found in 6.1% of the 163 persons and showed no association with hepatitis B infection (Table 1).

Table 1. Glucose-6-Phosphate Dehydrogenase Deficiency  
and Evidence of Hepatitis B Virus Infections

Subjects	Number	Number	G6PD Deficiency-%
HB <sub>S</sub> Ag+	18	1	3.2
Anti-HB <sub>S</sub> +	45	1	5.6
Total	63	2	3.2
Not HBV Infected	100	8	7.8

This project is now completed.

28. An Assessment of the Reverse Passive Hemagglutination  
as a Method for the Detection of Hepatitis B  
Surface Antigen

OBJECTIVE: To evaluate the sensitivity of the reverse passive hemagglutination (RPHA) test for the detection of hepatitis B surface antigen (HB<sub>S</sub>Ag).

BACKGROUND: According to recent evidence, a relationship may exist between hepatitis B virus (HBV) and chronic or malignant liver disease. Identification of the markers of HBV infections, therefore, become increasingly important. The RPHA test is reported to be a sensitive and simple method for detection of HB<sub>S</sub>Ag in serum.

METHODS: Comparative HB<sub>S</sub>Ag assays performed on sera collected from an asymptomatic Thai population employed the RPHA, immunoelectroosmophoresis (IEOP) and the radioimmune assay (AUSRIA-2, Abbott Laboratories, North Chicago, Ill.) A subsequent study was conducted on sera that had been shown to contain HB<sub>S</sub>Ag by AUSRIA-2. The RPHA test were obtained from commercial sources (Organon Co.) under the trade name of Heparosticon.

RESULTS: HB<sub>S</sub>Ag was detected in 18 of 100 sera by the AUSRIA-2 but was found in only five of 100 by the RPHA or the IEOP tests (Table 1). Of the 12 sera that were HB<sub>S</sub>Ag positive by AUSRIA-2, only two were shown to contain HB<sub>S</sub>Ag by the RPHA test (Table 2).

Table 1. Comparison of Three Tests for HB<sub>s</sub>Ag Results of 100 Sera

Test	Pattern of Positive Results		Sera Positive for each Test
AUSRIA-2 <sup>a</sup>	X	X	13
RPHA <sup>b</sup>	X		5
IEOP <sup>c</sup>	X		5
Total sera positive by tests indicated	5	8	13

a Radioimmune assay confirmed by neutralization

b Reverse passive hemagglutination

c Immunoelectrophoresis

TABLE 2

Sensitivity of RPHA for Detection of HB<sub>s</sub>Ag  
in Twelve Sera Positive by RIA Only

Test	Pattern of Positive Results	Sera Positive for Each Test
AUSRIA-2 <sup>a</sup>	X      X	12
RPHA <sup>b</sup>	X	2
Total sera positive by test indicated	2      10	12

<sup>a</sup> Radioimmunoassay confirmed by neutralization

<sup>b</sup> Reverse passive hemagglutination

TABLE 3  
Comparative Assessment  
of Different Test for Detecting HB<sub>S</sub>Ag

	IEOP	RPHA	RIA
Sensitivity	Adequate	Better	Best
Initial costs	Moderate	Low	Very high
Cost per test	Low	High	High
Time per test	2 hours	3 hours	5 hours
Reagent stability	Indefinite	6 months	30 days

On the basis of the sensitivity, specificity, economics, time and reagent stability of the three tests, the IEOP assay appeared the most useful for initial screening of sera for HB<sub>S</sub>Ag (Table 3). Confirmation of identity of antigen, however, must rely on the AUSRIA-2 test.

29. Continuing Studies of Transmission of Hepatitis B Virus to Gibbons by Exposure to Saliva Containing Hepatitis B Surface Antigen

OBJECTIVE: To determine if human saliva containing HB<sub>S</sub>Ag is capable of infecting gibbons by the oral route.

BACKGROUND: Previous attempts to demonstrate transmission of hepatitis B virus (HBV) to gibbons by the oral route, using saliva from HB<sub>S</sub>Ag carriers were unsuccessful (59). Investigations conducted in our laboratory and others (60) indicated that the risk of human infants acquiring HBV was greater for the offspring of mothers that possess high titers of hepatitis B surface antigen (HB<sub>S</sub>Ag) and hepatitis B e-antigen (HB<sub>e</sub>Ag). In view of this observation, attempts were made to demonstrate transmission of HBV by the oral route to gibbons, employing saliva from HB<sub>S</sub>Ag carriers who had high levels of HB<sub>S</sub>Ag and HB<sub>e</sub>Ag in their serum.

METHODS: Saliva was collected in a manner similar to that reported previously. One ml. of saliva was removed for testing. The remainder was promptly frozen at 70°C and pooled for transmission trials. Each one ml sample was tested for occult blood using paper strips impregnated with a buffered mixture of organic peroxide and ortholidine (Labstix, Ames Company, Ilkhart, Indiana).

The saliva used for transmission trials consisted of a pool of 10 specimens that were shown to contain HB<sub>S</sub>Ag by radioimmune assay. These specimens were collected from two HB<sub>S</sub>Ag positive donors who carried HB<sub>S</sub>Ag/adw at a level  $\geq 1:128$ . One female had transmitted HBV to her offspring (Table 1). Specimens were thawed rapidly, mixed together and centrifuged at 1,200 x g for 30 minutes at 4°C. After pooling the supernatant fluid of each specimen, tests were performed to determine if the mixture contained HB<sub>S</sub>Ag, occult blood and/or bacteria. Antibiotics, penicillin (1000 units/ml) and streptomycin (1000 mcg/ml), were added to saliva that was inoculated subcutaneously into monkeys. Results showed the saliva to contain occult blood, and to be positive for HB<sub>S</sub>Ag by radioimmune assay. All saliva specimens were maintained at sub-zero temperatures until used.

Gibbon management has been described earlier (59). Seven gibbons, 3 males and 4 females, with no detectable HB<sub>S</sub>Ag or antibody to HB<sub>S</sub>Ag were selected for exposure to the saliva pool (Table 2). Two, (B-40 and PC-26) received two subcutaneous injections of 2.5 ml on consecutive days. Two (P-16 and PC-16) of the five other gibbons were exposed on two consecutive days to 2.5 ml of the saliva pool over the same 2 day period. It may be assumed that the majority of the saliva administered by the oral and nasal routes was swallowed. Two additional gibbons, had anti-HB<sub>S</sub> resulting from previous inoculation with saliva that contained HB<sub>S</sub>Ag. These 2 gibbons were employed as controls for the antibody detection assay. The antibody control gibbons were not exposed to the saliva.

The first day of exposure to the saliva pool was designated Day 0. On every subsequent day, each animal was observed for altered behavior and the rectal temperature was recorded. Once a week, each gibbon was weighed and examined by a veterinarian. Following sedation with a rapidly acting intramuscular anesthetic (phencyclidine hydrochloride or ketamine hydrochloride), a blood sample was drawn for a complete blood count, serum transminase level (SGOT, SGPT), and hepatitis B serological tests.

All serum and saliva samples were tested for HB<sub>S</sub>Ag by solid phase radioimmune assay (AUSRIA II. Abbott Laboratories, Inc., North Chicago, Illinois) without preliminary treatment. Positive reactions were confirmed by a 50% or more reduction of test serum reactivity after incubation with a human serum containing anti-HB<sub>S</sub>. HB<sub>S</sub>Ag subtypes were detected by radioimmunoassay adsorption using subtype-specific rabbit antisera. Anti-HB<sub>S</sub> was detected by a solid phase radioimmune assay (AUSRAB, Abbott Laboratories, Inc.) interpreted according to the manufacturer's recommendations. Positive reactions were confirmed whenever possible by a 50% or more reduction in reactivity after incubating the test sera with human serum containing HB<sub>S</sub>Ag/adw.

RESULTS: HB<sub>S</sub>Ag was detected in the 2 gibbons (PC-26, B-40) inoculated with the saliva pool (Figures 1 and 2). PC-26 developed detectable antigenemia by the 10th week and carried it for 4 weeks (week 10-13) the SGPT rose towards the end of this period (week 13) and remained above background levels for 2 weeks. Anti-HB<sub>S</sub> was detected in samples taken on weeks 15-17 and from week 22 until the end of the study. HB<sub>S</sub>Ag was detectable in B-40 by the 16th week of the study. SGOT and SGPT were slightly

Table 1 Saliva Samples from Carriers of HB<sub>S</sub>Ag/adw used in the Saliva Pool

Donors	Transmit to Offspring	Serum		Saliva Samples			Contribution To Pool Amt. (ml)	Percent (%)
		HB <sub>e</sub> Ag (+)	HB <sub>S</sub> Ag Titer	Occult Blood Reaction	HB <sub>S</sub> Ag (+)			
Females (FM317)	+	+	1:181*	+	+		56	(68)
Male (SH)	?	+	1:128	+	+		25.5	(32)

\* Geometric mean titer over 4 samples

Table 2 Routes of Exposure of Gibbons to Human Saliva Pool  
from HB<sub>S</sub> Ag/adw Donor's

Exposure Category	Gibbon	Age(year)	Sex	Daily Dose	Day of Dosage	Total Dose
Subcutaneous Inoculation	B-40 PC-26	10 1.5	M F	2.5 2.5	D1,2 D1,2	5.0 5.0
Tooth brush & oral aerosol	P-16 PC-16	10 2.5	M F	2.5 2.5	D1,2 D1,2	5.0 5.0
Oral & Nasal aerosol	PC-21 PC-20	2 3	F M	2.5 2.5	D1,2 D1,2	5.0 5.0
	S-81	12	F	2.5	D1,2	5.0

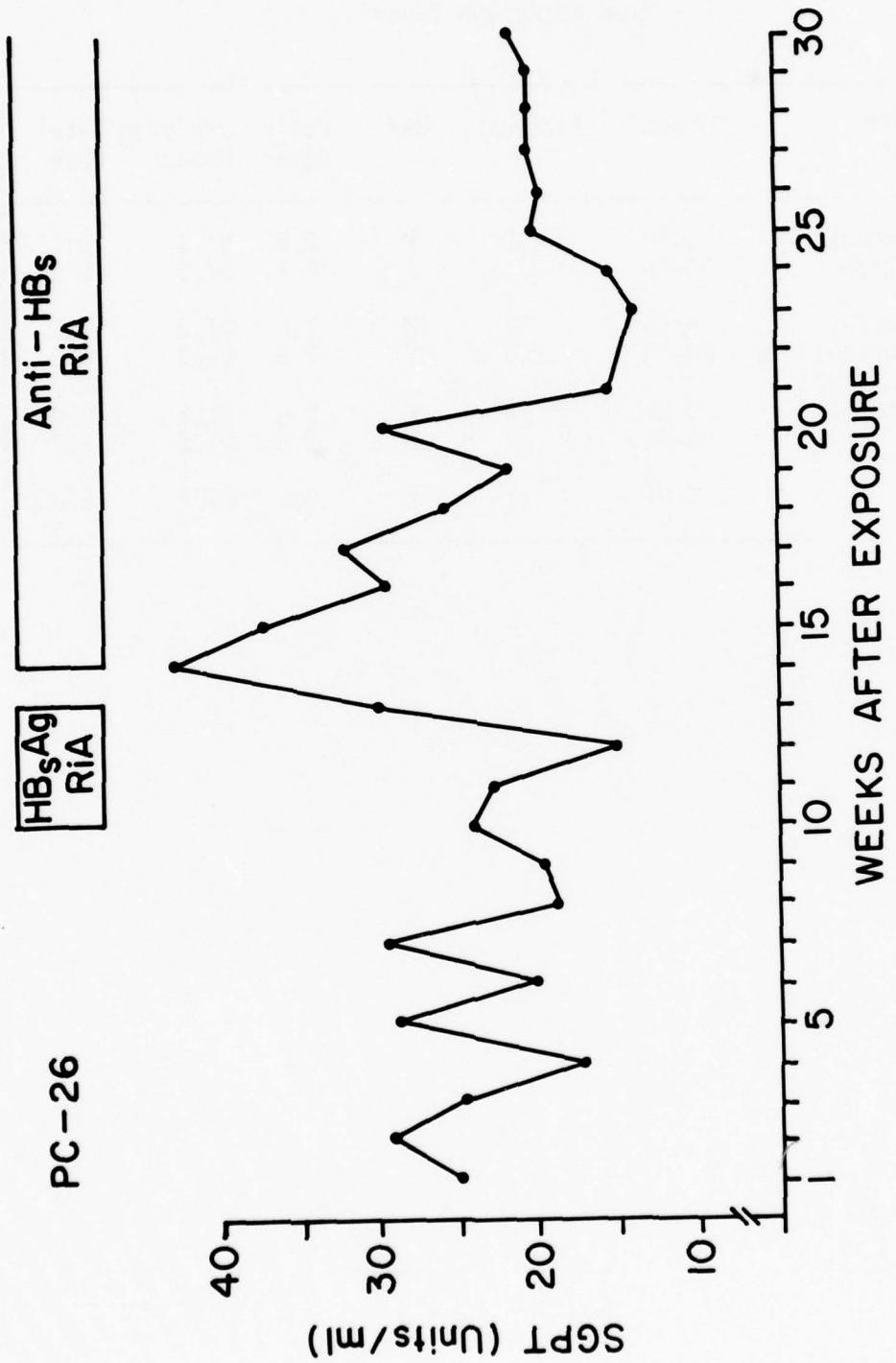


Figure 1. Response of gibbon PC-26 to the subcutaneous injection of a pool of human saliva containing HBsAg.

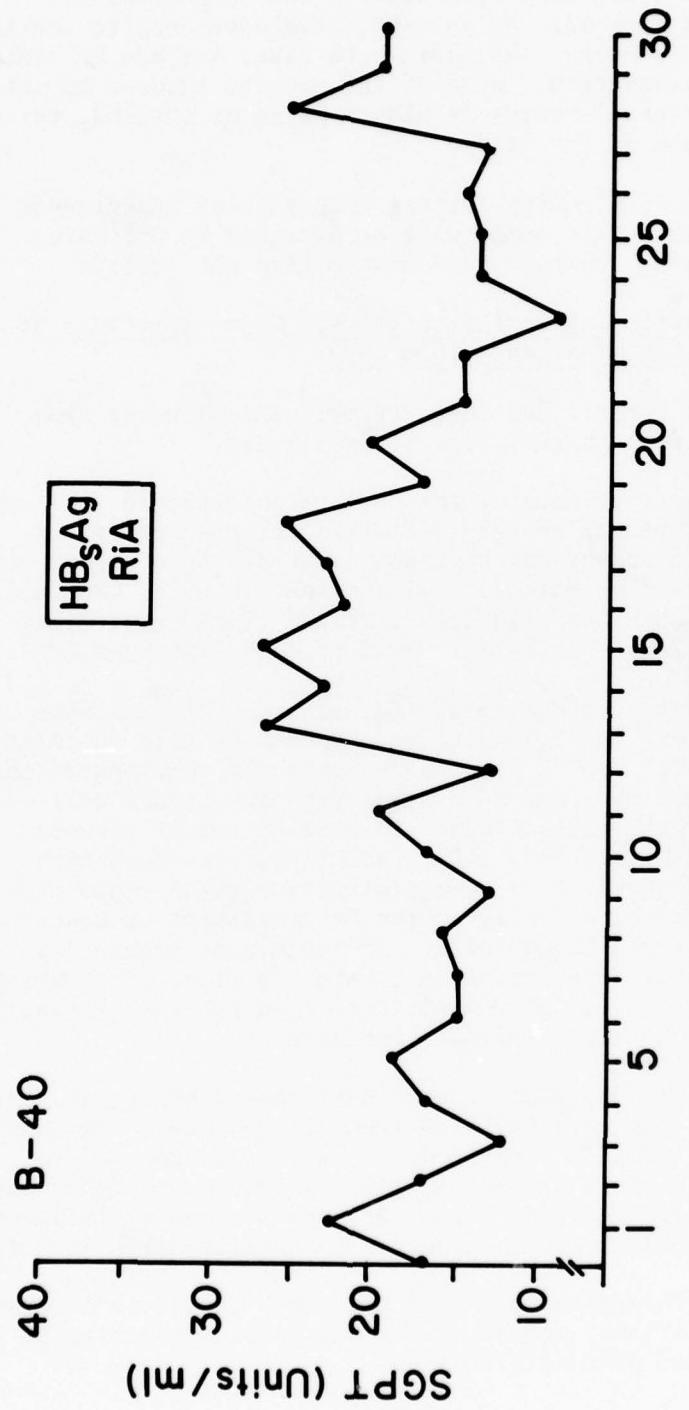


Figure 2. Response of gibbon B-40 to the subcutaneous injection of a pool of human saliva containing HB<sub>s</sub>Ag.

elevated above background from week 13-18. HB<sub>S</sub>Ag was not detected after week 18. No anti-HB<sub>S</sub> developed despite continued follow up for 12 weeks. HB<sub>S</sub>Ag in both cases was adw by radio-immune assay adsorption. None of the gibbons exposed to saliva by the oral or nasal routes developed HB<sub>S</sub>Ag or anti-HB<sub>S</sub> through the thirty weeks of the study.

The laboratory is planning further transmission experiments employing semen. This semen will be obtained by Dr. Harvey Alter from humans known to have transmitted HBV (61).

30. Isolation of Influenza Viruses From Residents of Bangkok, Thailand, 1976-1977

OBJECTIVE: To isolate and identify influenza viruses from patients with acute upper respiratory illness.

BACKGROUND: Surveillance of the human population for influenza viruses has proven to be very effective for predicting and combating epidemics caused by these viruses. This report consists of a summary of the isolations of influenza viruses obtained from patients who exhibited clinical signs and symptoms of upper respiratory illness during July 1976 through September 1977.

METHODS: Throat washings were obtained from patients with acute upper respiratory tract infections. Specimens were inoculated into the amniotic sac of nine to ten days old embryonated chicken eggs and/or onto monolayers of primary monkey kidney cell cultures. Techniques for the isolation and identification of viruses followed published methods (62). Briefly, virus isolation attempts were conducted by hemagglutination tests employing 0.5% chicken red blood cells and/or by hemadsorption tests using 0.4% guinea pig red blood cells. Antisera were prepared for virus isolates and prototype influenza strains in roosters. Subtypes of influenza virus isolates were identified by hemagglutination-inhibition tests using reference antisera.

RESULTS: During 1976 four viruses that caused hemagglutination of red blood cells were isolated from 21 specimens. The subtype of these four isolates were shown to be closely related to A/Victoria/1975. One of 35 specimens taken from patients with upper respiratory illness during 1977 one yielded an influenza-like virus. Studies are in progress to identify this isolate.

Isolates were forwarded to Walter Reed Army Institute of Research, to the CDC in Atlanta and/or to the WHO reference center in London for confirmation of identification.

Individuals Trained by Department of Virology, United States Component  
Armed Forces Research Institute of Medical Sciences  
July 1976 - September 1977

- | 1. | Dr. Surapee Seridhoranakul   | Bangkok Children's Hospital<br>Armed Forces Research Institute<br>of Medical Sciences, Research<br>Fellow             | Jul 76-Apr 77 | Research Methods   |
|----|------------------------------|---|---------------|--|
| 2. | Miss Pacharin Teinsarn       | Dept of Epidemiology, Armed<br>Forces Research Institute of<br>Medical Sciences, Thai Com-<br>ponent                  | Jul 76        | Hepatitis diagnostic<br>tests, tissue culture<br>methods |
| 3. | Dr. Raul H. Lopez-Correa     | Department of Health Education<br>and Welfare, Bureau of Laboratories<br>GPO Box 4532, San Juan, Puerto Rico<br>00936 | Aug 76        | Clinical aspects of<br>dengue hemorrhagic<br>fever       |
| 4. | Miss Pojana Seesawat         | Biological Science Division, Science<br>Department, Ministry of Public<br>Health                                      | Sep-Oct 76    | Complement fixation,<br>antigen preparation              |
| 5. | Mr. Watana Anuvanit          | Biological Science Division, Science<br>Department, Ministry of Public<br>Health                                      | Sep-Oct 76    | Complement fixation,<br>antigen preparation              |
| 6. | Miss Soontara Teowprasertkul | Dept of Microbiology, School of<br>Public Health, Mahidol University  | Oct 76        | Antigen preparation                                      |
| 7. | Mr. Suradej Dejkumwong       | Dept of Microbiology, School of<br>Public Health, Mahidol University  | Oct 76        | Antigen preparation                                      |
| 8. | Mr. Kachorn Chotilok         | Dept of Medicine, Siriraj Hospital<br>Mahidol University  | Nov 76-Feb 77 | Hepatitis diagnostic<br>techniques                       |
| 9. | Dr. Suttee Yoksan            | Dept of Pathology, Ramathibodi<br>Hospital, Mahidol University  | Dec 76-Jan 77 | Arbovirus serology,<br>viral isolation                   |

10. Dr. Chirasak Premsook Dept of Microbiology, School of Public Health, Mahidol University Arbovirus isolation, mosquito inoculation techniques Jan 77
11. 1st LT. Sukanya Kulanond Institute of Pathology, Royal Thai Army Hospital Hepatitis diagnostic techniques Jan 77-Feb 77
12. Mr. Soan Montathip Department of Epidemiology, Armed Forces Research Institute of Medical Sciences, Thai Component Glass washing techniques Feb 77
13. Mr. Somchai Wiriyayuthakarn Dept of Pathology, Prince Songkla University Hepatitis diagnostic techniques Mar 77
14. Miss Chanya Konlam Dept of Medicine, Siriraj Hospital Mahidol University Hepatitis diagnostic techniques Apr - May 77
15. Mr. Surina Chawanich Faculty of Science, Chulalongkorn University Hepatitis diagnostic techniques Jul 77
16. Mrs. Veena Cherdboonchard Graduate Student, Dept of Pathobiology Faculty of Science, Mahidol University Antigen preparation, HA, Jul 77-present HI, radioimmune assay techniques

### 31. Technical Training in the Department of Virology

OBJECTIVE: To serve as a training center for various techniques which are routinely performed in the Virology Department.

BACKGROUND: The Virology Department performs certain techniques which are considered useful by other institutions in Thailand. Therefore, we have developed a series of training programs in which trainees are taught the techniques required for certain tests and are then allowed to apply these techniques in practical situations.

METHODS: Written applications for training are received indicating the type of training required and the amount of time allotted for this training. If the training can be provided by the Virology Department, the trainee is assigned to the departmental section which is routinely performing the techniques. The trainee is placed with the technician performing the technique, the teaching technician describes the basic aspects and demonstrates the technique. The trainee then performs the technique until it is satisfactory to the teaching technician and the section supervisor.

RESULTS: Training performed by the Department of Virology is shown in Table 1.

### 32. Service Work of the Department of Virology

OBJECTIVE: To provide local institutions with laboratory information not available elsewhere in Thailand, using the specialized procedures routinely performed in the Virology laboratory.

BACKGROUND: The Virology Department in its research activities uses certain laboratory procedures which are not performed in most institutions. Upon request from outside organizations, the laboratory has undertaken a limited service function, providing studies for quality control purposes and for the clinical management of patients.

METHODS: In certain cases, specialized assays performed routinely in the Department of Virology were applied to specimens from non-protocol sources derived from inside or outside of the laboratory. These assays were broken down into six groups for the purposes of analysis.

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Table 1. Non-Protocol Studies Provided by the Department of Virology

	Rajavithi Hospital of Tropical Med.	Hospital Police	Children's Hospital	Royal Army Hospital	ChitengMai Hospital	AFRIMS Support	Other <sup>a</sup>	Total
Arbovirus Isolation	-	-	32 <sup>b</sup> (71.1)	6 (13.3)	-	4 (8.9)	3 (6.6)	45
Arbovirus Serology	12 (1.1)	23(5.6)	57(15.3)	164(40.1)	58(14.2)	41(10.8)	43(10.5)	11(2.7)
Non-Arbovirus Isolation	2 (1.1)	5(2.8)	3 (14)	93(52.2)	6(3.4)	9(5.1)	41(23.0)	19(10.7)
Non-Arbovirus Serology	1 (0.5)	5(2.7)	2 (1.1)	44(23.5)	70(37.4)	32(17.1)	16(8.6)	17(9.1)
HBsAg Assay(IEOP <sup>c</sup> )	3 (0.3)	4(0.4)	-	9(0.9)	934(90.4)	-	-	83(8.0)
Immunological tests	-	-	1 (1)	99(98)	-	-	1 (1)	101
Total	18 (0.9)	37(1.9)	62 (3.2)	343(17.6)	1173(60.1)	82(4.2)	104(5.3)	134(6.9)
								1953

<sup>a</sup> Includes the American Embassy Medical Unit, The Prince of Songkla University, and occasional requests from other Institutions

<sup>b</sup> Number of tests (percent of total)

<sup>c</sup> Includes Influenza Surveillance

<sup>d</sup> Majority of samples from the National Blood Bank, Central Institute of Pathology, Royal Thai Army Hospital Performed with technical help provided by the National Blood Bank.

<sup>e</sup> Immunolectric Immunoprecipitation.

1. Arbovirus isolation (dengue and chikungunya isolations)
2. Arbovirus serology (dengue, Japanese encephalitis virus and chikungunya serologies)
3. Non-arbovirus isolation (largely polio and influenza isolations)
4. Non-arbovirus serology (largely polio serology)
5. Hepatitis B surface antigen assay (screening by the Immunoelectroosmophoresis test)
6. Immunological tests (radial immune assays for IgG, IgM and complement factors)

RESULTS: A breakdown of the tests performed by the Department of Virology on non-protocol specimens is shown in Table 1. The Department has provided significant clinical or quality control services to six institutions outside of the laboratory as well as occasional services to the American Embassy Medical Unit and other organizations throughout Thailand. The laboratory honored a request in January 1977 by the National Blood Bank, Central Institute of Pathology, Royal Thai Army Hospital, to train personnel in techniques for the detection of Hepatitis B surface antigen ( $\text{HB}_s\text{Ag}$ ), with the help of these trainees, the laboratory provided  $\text{HB}_s\text{Ag}$  screening on blood donors' sera until the tests could be commenced in the Blood Bank.

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE AND TROPICAL DISEASES

Work Unit 008 Tropical and subtropical diseases in military medicine (SEATO)

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1979

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>2</sup> DA OB 6525	2. DATE OF SUMMARY <sup>2</sup> 77 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY <sup>3</sup> U	6. WORK SECURITY <sup>4</sup> U	7. REGRADING <sup>5</sup> NA	8. DISSEM INSTN <sup>6</sup> NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: <sup>7</sup> PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
6. PRIMARY	62770A	3M762770A802		00	009		
B. CONTRIBUTING							
C. CONTINUOUS		CARDS 114F					
11. TITLE (Precede with Security Classification Code) (U) Anti-schistosomal Drug Development							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>8</sup> 012600 Pharmacology 002600 Biology 010100 Microbiology							
13. START DATE 73 07	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-House		
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS			20. FUNDS (In thousands)
B. DATES/EFFECTIVE: NA		EXPIRATION:		FISCAL YEAR	77 CURRENT	1.1	30
D. NUMBER: <sup>9</sup>		E. AMOUNT:		FISCAL YEAR	78	1.6	36
C. TYPE:		F. CUM. AMT.					
G. KIND OF AWARD:							
21. RESPONSIBLE DOD ORGANIZATION							
NAME: <sup>10</sup> Walter Reed Army Institute of Research							
ADDRESS: <sup>11</sup> Washington, D C 20012							
RESPONSIBLE INDIVIDUAL							
NAME: RAPMUND, Garrison, COL							
TELEPHONE: 202-576-3551							
22. GENERAL USE							
Foreign intelligence not considered							
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Brazil; (U) Drug Development; (U) Schistosomiasis; (U) Chemotherapy							
24. TECHNICAL OBJECTIVE, <sup>12</sup> 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) Find new prophylactic and curative drugs which can be used to prevent and cure schistosomiasis infections that would be acquired by U. S. military and DOD civilians in the event of deployment in endemic areas such as South America, Caribbean, Africa, Middle East and Far East.							
24. (U) The WRAIR Anti-Schistosomal Drug Development Program submits drugs for prophylactic and therapeutic testing against schistosomiasis mansoni in mice. The prophylactic mortality test screens drugs against mice exposed to 3,000 or more S. mansoni cercariae, and the drugs are given subcutaneously in a single dose at 1280 mgs/kg. The curative test uses mice exposed to 200 cercariae, and 30 - 35 days later, drugs are given subcutaneously at 100 mgs/kg for five days. Prophylactic drug activity is measured by mouse survival and curative drug activity is measured by sick and dead worms in the liver.							
25. (U) 76 10 - 77 09 This research is complementary to studies being conducted under DAOB 7294, Work Unit 086, entitled Chemotherapeutic Studies on Schistosomiasis. The laboratory Biomphalaria glabrata (Paulista) snail colony maintained an average daily level of 1,318 S. mansoni cercariae shedding snails. The miracidia exposed snail's survival rate was 88 percent with a 52 percent infection rate. Thus, 3 to 5 million cercariae were available for weekly mouse exposures. A significant breakthrough occurred in our drug testing program with the mouse bedding change over from wood chips to ground corn cobs, the mouse group infection failures dropped from 30 percent to zero. During FY77, 2,096 selected WRAIR bottle number drugs were tested for prophylactic and curative activity. The results were as follows: A. Prophylactic testing, 1) 879 drugs negative, 428 drugs toxic, and 3 drugs active; B. Curative testing, 1) 704 drugs negative, 41 drugs toxic, and 41 drugs active. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.							

DD FORM 1498  
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68  
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

1071

Project 3M762770A802 TROPICAL MEDICINE

Work Unit 009 Anti-Schistosomal Drug Development

Investigators.

Principal: Myron G. Radke, LTC, MSC

Associate: Aluizio Prata, M.D. ( University of Brasilia )

Description.

It is an appalling fact that schistosomiasis remains one of the few tropical diseases in which there exists no backup drugs for treatment. The few drugs marketed, Niridazole, Hycanthone, and Oxamniquine, give about an 80 percent cure rate but are accompanied by some major side-effects. At no time in our medical history have we been so vulnerable to a tropical disease as schistosomiasis because prophylactic drugs are non existent and curative drugs are limited. Today's global strategic areas are Africa and the Middle East which are hot beds of schistosomiasis. A major research effort in the anti-schistosome drug development program is being carried out by the U. S. Army Walter Reed Army Institute of Research. The drugs are obtained from the Division of Medicinal Chemistry (WRAIR). These drugs are tested for prophylactic and curative anti-schistosomal activity in mice at the U. S. Army Medical Research Unit/Brasilia. Our research is aimed exclusively towards finding prophylactic and curative drugs for use in the prevention and treatment of schistosomiasis.

Progress.

a. Laboratory Facility. Although this unit was designed and equipped to test drugs, we have upgraded the overall equipment giving us limited general laboratory capability by having on hand: 1) portable sterilizer, 2) 12 place centrifuge, 3) constant temperature box, 4) oven, and 5) a lyophilizer. We have a single recurring problem with the air compressor that provides oil/water free air for the laboratory snail colony. The United States made ITT air compressor has had a good deal of down time. It is impossible for us to predict the operating time ( one month to six months ) of the teflon coated compressor heads which overheat and cause the cooling fans to destruct. Even though we have sufficient compressor units to obtain stateside servicing, the maintenance costs are high and it maybe necessary in the near future to change over to a Brazilian made air compressor.

b. Animal Facilities. Almost all laboratories through out the world use a woodchip or sawdust type bedding to house mice. In our early years of anti-schistosome drug testing in Japan, 1964, we also had a 30 to 40 percent mouse test group failures in which the mouse infections were not up to the standards set and the entire test group results had to be discarded. Even until very recently, 1976, the experimental test group's control infected mice in the primary mortality test system had a 30 percent failure rate for mice exposed to 3,000 or more S. mansoni cercariae but failed to give an LD<sub>99</sub> by the 30th day of infection. In July 1976, we suspected that the infection failures might be caused by toxic substances present in the woodchip bedding. Our analysis of the woodchip bedding did show that substances were present that affected the infection in mice. During October 1976, we changed over from woodchip to ground corn cob bedding for mouse maintenance. The entire breeding colony at the Bioterio of the University of Brasilia and our drug test animals were placed upon ground corn cob bedding. Since the change over in mouse bedding, we have had no schistosomiasis mouse test group failures. We now know that of the 20 Brazilian woods tested that at least three have toxic properties to mice and even man ( allergic responses ). In our opinion, if schistosomiasis research laboratories are experiencing poor infections in mice, the initial problem to address is the bedding rather than an S. mansoni cercariae problem.

c. Snail Colony. The laboratory Biomphalaria glabrata snail colony ( Paulista Strain ) provides a uniform source of S. mansoni cercariae for infecting mice. The snail colony is responsive to our cercarial usage, and our daily average on hand number of cercariae shedding snails was 1,318 for the FY 77. We expose individually 400 snails weekly to 8 to 10 S. mansoni miracidia that are recovered from macerated infected Tivers from the schistosome life cycle mice. During the snail's 42 day incubation period, the snail survival rate was 88 percent and of those surviving snails 52 percent were isolated as positive cercarial shedding snails. The snail facility's schistosome mansoni cercarial production is at 5 million cercariae per week ( sufficient cercariae to expose 1,250 mice weekly to 4,000 cercariae ).

d. Drug Testing. The 500 to 1,000 mg drug samples received allows us to carry out both prophylactic and curative testing for anti-schistosome activity. We test 50 drugs twice monthly for prophylactic activity by the mouse mortality test system. Drugs are given subcutaneously at 1280 mgs/kg on the second day after exposure to 3,000 or more S. mansoni cercariae. In the curative test, 60 drugs are tested twice monthly. Each drug is given subcutaneously at 100 mgs/kg for five consecutive days beginning 33 to 35 days after mice were exposed to 175/200 S. mansoni cercariae.

e. Schistosomiasis. A series of experiments were carried out to standardize the Paulista Strain of schistosomiasis mansoni in mice for the mortality and the curative test systems. Mice were exposed to seven cercarial doses ( 500, 1000, 1500, 2000, 2500, 3000, and 3500 ) with four exposure times ( 15, 30, 45, and 60 minutes ). The mouse deaths were recorded daily. Likewise, mice were exposed to 75, 150, and 225 cercariae for 15, 30, 45, and 60 minutes. The infection rates were determined by mouse worm burdens. The preliminary data shows that the death rates and the mouse worm burdens are about the same for the four different exposure times.

f. Operating Personnel. The drug testing program is directed by one Senior American Investigator and supported by a staff of eight Brazilian Laboratory Assistants ( one position vacant ). The operating program is broken down into five work areas which are: 1) Snail Colony ( 2 people ), 2) Animal Service ( 2 People ), 3) Necropsy ( 1 person ), 4) Pharmacy ( 2 people ), and 5) Administrative ( 1 person ). However all individuals are cross trained to perform the 7 day work schedule of daily snail maintenance, subcutaneous and gavage drug administration, daily mouse maintenance with mortality checks, and mouse exposures to cercariae. Besides each individual is able to perform all duties in two other areas of work. The drug testing facility staff members are:

Director: LTC Myron G. Radke

Laboratory Assistants:

Kazuyoshi Ofugi  
Harley Azevedo Junior  
Joao Bosco Ferreira de Araujo  
Shigeru Ofugi  
Altamir Jose Rodrigues  
Joao Dalmacio Barros da Silva  
Walcymar Pereira Santiago  
Vacant

Test Procedure.

Routinely all drugs received are first screened for prophylactic anti-schistosomal activity by the mouse mortality test system (Radke, et. al., 1971), and secondly the drugs are tested for curative activity unless otherwise directed by the Division of Medicinal Chemistry (WRAIR). The mice used in both test systems are the Swiss-Holland 40 albino mice,  $43 \pm 5$  days, weighing 18-23 grams.

The mortality test system evaluates drugs for prophylactic activity. Mice are tail exposed individually to 3,000 or more S. mansoni

cercariae for 45 minutes. Two days after cercarial exposure, the drugs are given subcutaneously in a single inoculation at 1280 mgs/kg to five test animals. Active drugs are identified by mouse survival. Each prophylactic test group uses 315 mice, 250 infected mice are used to screen 50 drugs, 50 infected nontreated control mice, five Niridazole treated mice ( reference drug ), and 10 normal mice. The infected nontreated control mice will begin dying on the 20th day of infection and none will survive beyond the 30th day. Active drugs are those in which mice survive two weeks after all infected control mice are dead. At 49 days, all surviving mice are sacrificed and perfused ( Radke, et. al., 1961 ) for total worm burdens.

In the curative test system mice are tail exposed individually to 175/200 *S. mansoni* cercariae for 45 minutes. Thirty-three to 35 days later the mice are given drugs subcutaneously at 100 mgs/kg for five consecutive days. Two days after treatment is completed, the mice are killed individually, livers removed, and squashed between two glass plates. The dead/live worm counts are made from the liver squash preparation as read under a dissecting microscope. Each curative test calls for 340 mice, 300 infected mice are used to screen 60 drugs, 15 infected mice treated with Oxamniquine ( reference drug ), 15 infected control mice, and 10 normal non-infected mice. An active drug is one in which the live worm counts are 2 to 3 times greater than the infected nontreated control mice or dead worms are found.

### Results.

During FY 1977, a total of 2,096 bottle number drugs were tested by both the prophylactic and curative test systems. Using the mortality test, 1,310 bottle number drugs were tested either at 1280 mgs/kg or at a lower retest dosage of 40 and 160 mgs/kg. The prophylactic test results were: 879 drugs were negative, 428 drugs were toxic, and 3 drugs were active. In the curative test, 786 bottle number drugs were screened for anti-schistosome activity at either 100 mgs/kg for five days or at a lower dosage. Drug test results were: 704 negative, 41 toxic, and 41 active. The curative test is very sensitive to drug action as reflected by the live worm shift to the liver and/or the presences of sick/dead worms. By means of both test systems, 42 bottle number drugs were found active. The chemical structures of these active drugs are restricted since the active compounds are all "Discrete" drugs. Discrete compounds are protected under U. S. Laws. These are compounds furnished by Commercial Companies for anti-schistosome testing before they have secured a registered U. S. Patent on the drug.

Project 3M762770A802 TROPICAL MEDICINE

Work Unit 009 Anti-Schistosomal Drug Development

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup> DA DB 6528	2. DATE OF SUMMARY <sup>b</sup> 77 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMMARY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCY <sup>c</sup> U	6. WORK SECURITY <sup>d</sup> U	7. REGRADING <sup>e</sup> NA	8. DGS/BN INSTR'N NL	9a. SPECIFIC DATA - CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: <sup>f</sup> a. PRIMARY 62770A	PROGRAM ELEMENT PROJECT NUMBER 3M762770A802			TASK AREA NUMBER 00	b. LEVEL OF SUB a. WORK UNIT WORK UNIT NUMBER 010	
b. CONTRIBUTING 9X9XX9X9X9X9X9X	CARDS 114F					
11. TITLE (Pencils with Security Classification Code) (U) Disease Transmission in Tropical Populations.						
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>g</sup> 010100 Microbiology 002600 Biology 005900 Environmental Biology.						
13. START DATE 73 11	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House			
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS		
a. DATES/EFFECTIVE: NA EXPIRATION:		FISCAL YEAR	77 CURRENT	5	b. FUNDS (in thousands) 150	
b. NUMBER: <sup>h</sup>			78	4	150	
c. TYPE:		d. AMOUNT:		e. CUM. AMT.		
e. KIND OF AWARD:						
19. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research		20. PERFORMING ORGANIZATION NAME: US Army Medical Research Unit Belem (Transamazon)		PRINCIPAL INVESTIGATOR (Pencil BMAN if U.S. Academic Institution) NAME: ROBERTS, D.R., MAJ., MSC.		
ADDRESS: Washington, DC 20012				TELEPHONE: SOCIAL SECURITY ACCOUNT NUMBER: ASSOCIATE INVESTIGATORS NAME: HOCH, A. L., CPT., MSC. NAME:		
21. GENERAL USE Foreign intelligence not considered.						
22. KEYWORDS (Pencils EACH with Security Classification Code) (U)Brazil; (U)Infectious Disease; (U)Epidemiology; (U)Entomology; (U)Parasitology; (U)Virology; (U)Serology.						
23. TECHNICAL OBJECTIVE, <sup>i</sup> 24. APPROACH, 25. PROGRESS (Pencil individual paragraphs identified by number. Pencils text of each with Security Classification Code.)						
23(U) Conduct epidemiologic studies of infectious diseases in populations along the Transamazon highway and studies on the ecology of Oropouche virus transmission in the Amazon basin in collaboration with the Evandro Chagas Institute. Information regarding health hazards, such as Oropouche virus, is important to military personnel transiting or stationed in the geographic area.						
24(U) Routine diagnostical, epidemiological, entomological, microbiological, virological, and serological procedures are employed. Consists of integrated field and laboratory studies with emphasis on field surveillance along the Transamazon highway and at areas near the cities of Santarém and Belém.						
25(U) 76 10-77 09. The surveillance program along the highway was terminated during this report period and studies on Oropouche virus were initiated. Malaria was found to be a serious problem among migrant laborers and many cases of falciparum malaria did not respond to the standard treatment with chloroquine. Indices of faunal diversity for disease vectors and reservoirs have been calculated from field data. The house frequenting habits of several potential disease vectors have been studied, with emphasis on vectors of malaria. Oropouche virus attack rates of 40% have been documented during urban epidemics. Culicoides paraensis and Culex quinquefasciatus have been incriminated on epidemiological grounds as vectors of Oropouche virus during urban epidemics. Culicoides paraensis have efficiently transmitted the virus to animals in the laboratory. This species aggregates near houses while seeking blood meals and its peak in biting activity is 1700-1800 hr. The other candidate as vector, Culex quinquefasciatus, does not appear capable of efficiently transmitting the virus under laboratory conditions. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1976 - 30 Sept 77.						
Available to contractors upon originator's approval.						

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1977

PROJECT 3M762770A802 TROPICAL MEDICINE

TASK 00, Tropical Medicine

Work Unit 010 Disease Transmission in Tropical Populations

**Investigators**

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ANNUAL REPORT      USAMRU-BELEM

INTRODUCTION

The program of epidemiological surveillance conducted along the Transamazon highway, Para, Brazil from 1974-1976, was terminated during this work period. Some of the data from the program will be reported here; however, much of the epidemiological, entomological and ecological data remain to be collated and analyzed.

Interesting observations on urban epidemics of Oropouche virus were made during the surveillance program, see Progress Report for 1976, and a continuing program of studies on the vectors and natural reservoirs of the virus in the Amazon region was initiated during FY 77.

1978

I. MULTIDISCIPLINARY DISEASE SURVEILLANCE ALONG THE TRANSAMAZON HIGHWAY

A. EPIDEMIOLOGY - MALARIA IN MIGRANT LABORERS

OBJECTIVE: To describe the epidemiology of malaria in migrant laborers and assess the effectiveness of malaria chemotherapy.

BACKGROUND: Migrant laborers were commonly employed by colonists along the Transamazon Highway. Their presence in the study area stimulated the undertaking of a study to obtain information on the prevalence of malaria in migrant worker populations. The target population included in the study were workers on a fazenda located in the area of Paragominas, Pará, Brazil; 150 Km south and 195 Km east of Belém, Pará, Brazil. This location is at the junction of the Uraim and Gurupi rivers. Surveillance methods, both active and passive, of workers for malaria were described in the Annual Report for 1976.

DESCRIPTION: All persons with slide-proven malaria were confined for treatment. The cases were isolated in a treatment facility located more than 1 Km from other living quarters. Treatment regimes were prepared in general compliance with the SUCAM (Superintendência Campanha Saúde Pública) recommendations. Cases of falciparum malaria were treated on day 1 with 600 mg and days 2 and 3 with 400 mg each of chloroquine phosphate. Slides were read daily for each patient. Patients positive with asexual blood forms on day 3 were subsequently treated for 2 days with a drug combination. The 1st day the patient received 50 mg of pyrimethamine and 1000 mg of sulformetoxine. Only 500 mg of sulformetoxine was administered during the 2nd day of treatment. All positives after day 2 were given another 2 day treatment regime of pyrimethamine or sulformetoxine. Quinine sulfate was employed for all cases that did not respond to the 2nd cycle of treatment.

Cases of vivax malaria were treated with chloroquine phosphate, 600 mg on day 1 and 450 mg on days 2 and 3. Afterward, the patient was given 15 mg of primaquine daily during the next 14 days

Patients were maintained in isolation during the treatments with chloroquine and pyrimethamine-sulformetoxine medications. Medication was administered individually and each patient was observed to see that the medication was taken. After each patient had completed their respective treatment regimes they were released and returned to the work areas. The primaquine and quinine medications were dispensed daily in the field. Records

on treatment and treatment effectiveness were maintained on each patient.

PROGRESS: Men represented 91% of the fazenda population and about 63% were between the ages of 15-29 years (Table 1). The age-sex distribution reflected a large number of young single men and a relatively small complement of fazenda families.

Analyses of population based data revealed no significant differences in attack rates by sex or age groups. Observations were compiled on individual workers to determine the sequence of case occurrence vs time on the fazenda. The majority of cases occurred in the 1st sequential visit, i.e., within the 1st 2 weeks after arrival at the fazenda (Table 2).

A considerable number of malaria cases were imported by workers entering the fazenda from other areas. Between March and May, 1976, 588 workers were examined at the fazenda gate. Sixty-five persons (11.05%) were slide positive for malaria. Thirty-two persons (5.44%) had falciparum malaria and 31 persons (5.27%) were positive for P. vivax (Table 3). The Paragominas area provided the largest number of job applicants (41%) as well as the highest positivity rate among applicants (18%).

A considerable number of cases of falciparum malaria did not respond to chloroquine therapy. Especially notable are the large percentages of falciparum cases that remained slide positive after 3 days of chloroquine treatment (Table 4). Detailed observations on 281 falciparum cases indicated only 56% responded to a 3 day regime of chloroquine treatment (Table 5). Inspection of the records for each of the 281 individuals indicated that recrudescence of falciparum malaria was recorded for 9 of the patients. These 9 cases were within 21 days of release from the treatment facility. Only 1 recrudescence was detected in cases treated with the pyrimethamine-sulformetoxine combination. A comparison of effectiveness of chloroquine vs pyrimethamine-sulformetoxine, by chi-square analysis, indicated a significant ( $p < 0.01$ ) difference in drug effectiveness (Table 6). Observations from the 1st visits indicated that 50% of falciparum malaria recrudescences were detected in the next biweekly visit following treatment (Table 7).

COMMENT: Malaria is definitely a problem in migrant labor forces in the area of Paragominas, Pará, Brazil. Studies should be undertaken to determine the prevalence of malaria in migrant populations in other areas of the Amazon basin. The available data indicate that the standard treatment regime for falciparum malaria is not always effective.

Table 1

Age-sex Distribution of the Migrant Worker Population on a Fazenda Near Paragominas, Pará, Brazil. Personnel Included in 10 Biweekly Surveys in 1976.

Age Group	Numbers of Individuals	
	Males	Females
0 - 4	32	29
5 - 9	26	21
10 - 14	24	15
15 - 19	358	17
20 - 24	398	26
25 - 29	350	22
30 - 34	166	18
35 - 39	73	6
40 - 44	81	4
45 - 49	40	0
50 - 54	27	0
55 - 59	15	1
60 - 64	4	0
65 - 69	0	0
70 - 74	0	0
75 - 79	2	0
> 80	0	0
Total	1,596	159

/zcm.

Table 2

Distribution of Case Occurrence by Sequence in Biweekly Malaria Surveys (visits) from Observations on Individual Workers on a Fazenda Near Paragominas, Pará, Brazil in 1976.

Visit	Number of Cases*	
	Falciparum Malaria	Vivax Malaria
1	92	60
2	79	30
3	53	34
4	13	9
5	12	13
> 5	13	8
Total	262	154

\* Each positive case listed according to time individual 1st appeared in 2-week visits and subsequent visit in which individual was slide positive. Individuals in the 1st of the 10 visits were not included.

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Table 3

Malaria Positivity rates in Migrant Workers, by Origins, on Arrival at a Fazenda Near Paragominas, Pará, Brazil in 1976.

Origin of Applicants	Number of Applicants	% Slide Positive		Total % Pos.
		Falciparum	Vivax	
Paragominas area	242	8.3	10.3	18.6
Imperatriz	29	6.8	3.4	10.2
Belém	48	4.2	0	4.2
Piaui	20	10.0	5.0	15.0

/zcm.

Table 4

Percentage of Slide Positive Cases of Falciparum Malaria After 3 Days of Chloroquine Treatment, February-August, 1976. Patients Were From a Fazenda Work Force Near Paragominas, Pará, Brazil in 1976.

Month	Number of Cases	% Positive After Treatment
February	92	43.5
March	122	33.6
April	61	42.6
May	26	7.7
June	55	34.5
July	88	32.9
August*	14	35.7

\* Data incomplete

/zcm.

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Table 5

Response of *Falciparum* Malaria to a Treatment Regime as Determined by Microscopic Examination for Asexual Parasites. Cases Were Detected in Migrant Workers at a Fazenda Near Paragominas, Pará, Brazil in 1976

	Days of Treatment													
	0	1	2	3	4	5	6	7	8	9	10	.....	14	
Chloroquine														
Pyrimethamine and Sulfometoxine														
Treatment regime														
Quinine Sulfate														
Number of cases treated	281													
Numbers of cases responding to treatment	160													
Percent of cases responding to treatment	56%													
	71%													
		65%												
			100%											

/zcm.

Table 6

Recrudescence of Falciparum Malaria Following Treatment With  
Chloroquine Phosphate or a Combination of Pyrimetamine and  
Sulformetoxine.

Treatment	Response to Treatment (Number of Cases)		Total
	Recrudescence	No Recrudescence	
Chloroquine Phosphate	8	152	160
Pyrimetamine and Sulformetoxine	1	120	121
Total	9	272	281

$$\chi^2 = p < 0.01$$

/zcm.

1096

Table 7

Distribution of Case Occurrence in Sequential Biweekly Malaria Surveys Following the 1st Case of Malaria in Each Person\*. Data From a Malaria Control Program Conducted on a Fazenda Near Paragominas, Pará, Brazil in 1976.

Visit Number**	Number of Cases	
	Falciparum	Vivax
1 <sup>a</sup>	22	2
2 <sup>a</sup>	9	2
3	5	0
4	4	0
5	2	0
6	1	0
7	0	3
8	1	1
9	0	0
10	0	0

\* Cross infections (vivax/falciparum) not included in these data.

\*\* Sequence of 2-week visits following the 1st case of malaria in each person.

<sup>a</sup> Reoccurrences of falciparum malaria cases that would generally be accepted as recrudescences (occur within 30 days after the 1st case).

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## B. MAMMAL SURVEILLANCE AND ECOLOGY

OBJECTIVE: Systematically collect, identify and enumerate the species of mammals present along the Transamazon Highway, Pará, Brazil, and to:

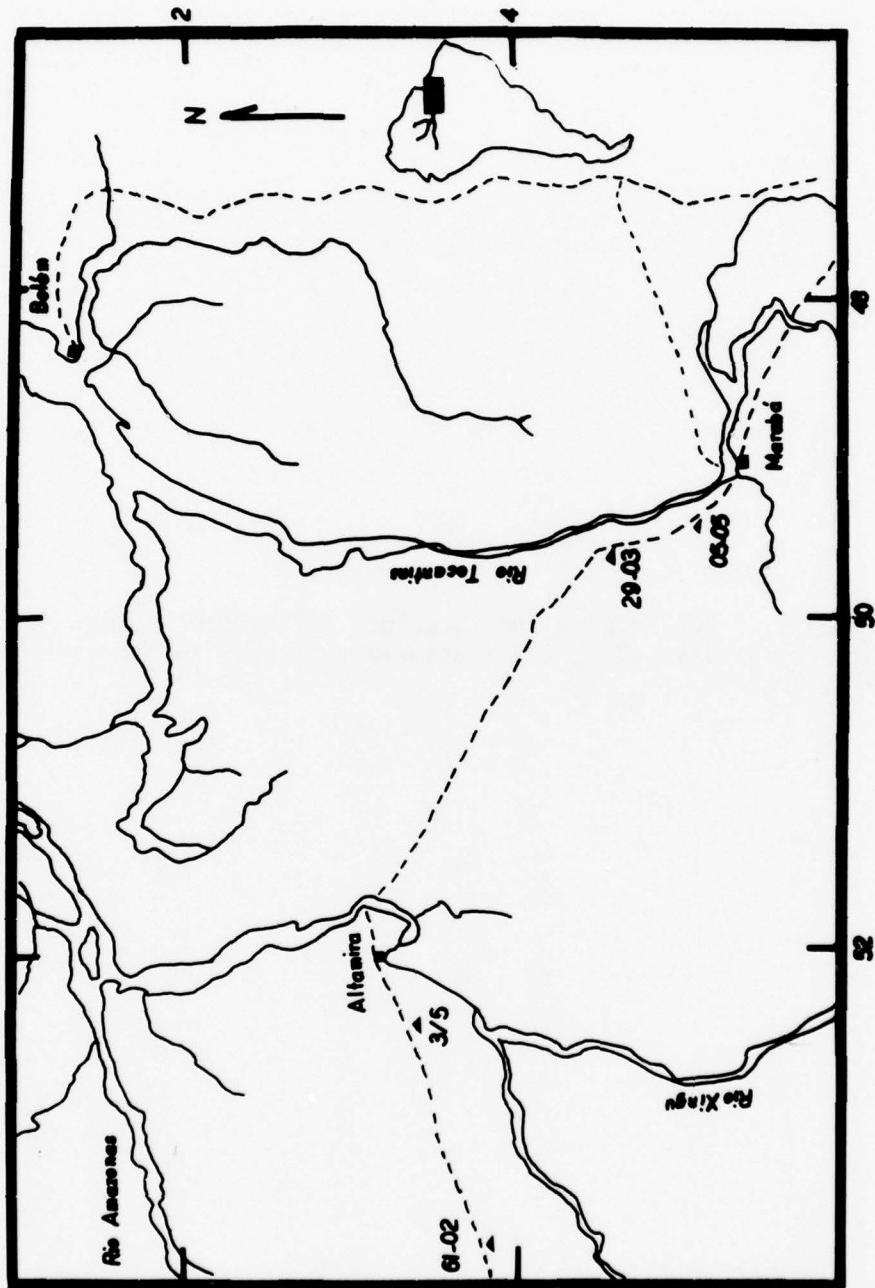
1. Document the prevalence of infectious disease agents in the mammal populations and thus, identify species most likely to serve as reservoirs of human disease.
2. Determine habitat preferences for various species and detect changes, in mammal populations, resulting from colonists' efforts to convert the primary forest to cropland.

BACKGROUND: The opening of the Transamazon Highway provided access to a variety of ecologically different areas. As the new areas were opened to colonization, the extremely rapid ecological changes caused by human invasion of the primary forest provided an opportunity for studies over a sufficient period of time to document resulting changes in mammal abundance and species composition. A surveillance program designed to monitor these changes was initiated in November 1974 and terminated in December 1976. The information gathered during this study, when compared with simultaneously collected epidemiological and entomological data from the same areas, will contribute to a better understanding of vector-reservoir interactions, and may assist in defining the presence and potential disease risks to humans from zoonoses.

DESCRIPTION: The mammals of the Transamazon region are highly diversified, and small changes in a habitat may result in profound changes in both the species composition and abundance in a region. In an attempt to monitor such changes, a trapping program was designed to record the relative abundance of various mammals found in ecologically diverse habitats.

Two collection sites were established in each of the Marabá and Altamira areas. These sites were separated by 1 large river, the Xingu, and a maximum distance of 570 Km (Fig. 1). These sites were selected for intensive surveillance to obtain a representative sample of the mammals present. Because of the importance of sampling different habitats, traps were systematically dispersed in cropland, orchards, secondary scrub, and in as many different forest types as possible. The "edge" between forest and disturbed areas was similarly covered. Trapping was conducted for 2 weeks of every month in each of the trapping sites. The types of traps used, trapping methods, area descriptions, and animal processing techniques have been

Figure 1. Map showing the locations of the four mammal collecting sites along the Transamazon highway in the state of Pará, Brazil.



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previously described in the Annual Reports for 1975 and 1976.

The blood, sera, blood slides, and organ specimens from captured mammals were preserved and shipped to the main laboratory in Belém. The sera were tested for Chagas disease, toxoplasmosis, leptospirosis, brucellosis, schistosomiasis, and virus antibodies in Belém, and for plague, tularemia, and rickettsial antibodies at the Division of Hazardous Microorganisms, WRAIR. The blood slides were examined microscopically for evidence of anthrax, microfilariae, trypanosomes, and malaria. The organs preserved in 10% formalin were sent to the Department of Veterinary Pathology, WRAIR for examination.

Few data are available comparing the species of mammals present and abundance of common species in different tropical forests which are physiognomically similar, but separated by natural barriers and distance. Such data were collected during the surveillance program along the Transamazon Highway. Two indices, the percentage of similarity and the quotient of similarity, were used to measure the similarities between habitats in composition of mammal species and relative abundance of the "common" species. The percentage of similarity is based on a comparison of the species make-up of 2 areas in terms of individuals of the various species, and it places the emphasis on the dominant or common species (Southwood, 1968). The index is obtained by summation of the lowest percentages of the species found in both samples (habitats):

$$\% S = \sum \min. \% (a, b, c, \dots, x)$$

The quotient of similarity places the emphasis on the rare species, as the number of species found in both habitats is most important:

$$QS = \frac{2j}{a+b}$$

Where  $j$  = the number of species in common,  $a$  = the number of species in habitat A, and  $b$  = the number of species in habitat B.

These indices were arrayed in trellis diagrams to illustrate the similarities in the mammal fauna between habitats. Associations were determined by placing the largest values on the diagonal line, thus habitats with highest similarities were placed together.

RESULTS:

Laboratory Examination for Disease Agents

From November 1974 through December 1976, 3,068 mammals were collected by trapping, mist netting and hunting. A panel of sera from 291 mammals collected along the Transamazon Highway were sent to WRAIR and were tested for plague, tularemia, and rickettsia antibodies. Sera were collected from 1,030 mammals (Table 1) and tested for Chagas disease, toxoplasmosis, leptospirosis, brucellosis, schistosomiasis, and viruses in the Belém laboratory. Blood smears from 2,032 mammals were examined for anthrax, microfilariae, trpanosomes, and malaria in Belém.

The results of the agglutination test for detection of antibodies against Trypanosoma cruzi (Table 2) indicated 37% (56/152) of the rodent sera tested from gleba 61 lote 02 were positive. This antibody prevalence rate was significantly ( $\chi^2 P > 0.995$ ) greater than that seen in rodents from the other trapping sites. Marsupials had a higher percentage of positivity in gleba 05 lote 05 ( $\chi^2 P \leq 0.99$ ) than either gleba 61 lote 02 or gleba 29 lote 03. Some sera reacted to the T. cruzi antigen in all mammal groups tested.

Sera were tested for antibody to Toxoplasma gondii using the indirect hemagglutination (IHA) test. Only 6 of 651 mammal sera tested were considered positive (titer  $\geq 1:256$ ) (Table 3). One marsupial (Didelphis marsupialis) produced a titer of 1:4096.

A total of 997 mammal sera were tested for Brucella by the rapid slide agglutination test, and 9 were found positive (Table 4). Of the 19 species of rodents tested, 2 species demonstrated antibodies to Brucella, the spiny rats, Proechimys longicaudatus with 5 positive sera of 150 tested, and P. guyannensis with 3 sera positive of 154 tested. All were collected in the forest. The positive bat (Sturnira tildae) was collected in the secondary scrub, but this species normally is forest dwelling. None of the terrestrial mammals collected in the secondary scrub or cropland areas were positive. Dogs, a few pigs, and 1 burro were the only domestic mammals present near the collecting sites. Cattle were present in gleba 29 lote 03, and were introduced only during the last 3 months of the project, at least 5 months after the positive spiny rats were collected.

The IHA test for schistosomiasis detected low antibodies titers in a number of mammal sera, but only 1 spiny rat (Proechimys guyannensis) collected in gleba 3/5 produced a titer as high as 1:256 (Table 5).

Table 1

Sera Available From the Mammal Surveillance Program by Type of Mammal and Location Along the Transamazon Highway. Collections Made From November 1974-December 1976.

Mammal Group	Altamira		Marabá		Total
	G 3/5	G61 L02*	G05 L05	G29 L03	
Marsupials	44	87	39	45	215
Bats	-	7	-	-	7
Monkeys	5	-	-	3	8
Edentates	2	-	2	2	6
Rodents	221	281	89	199	790
Carnivores	3	1	-	-	4
Total	275	376	130	249	1,030

\* Sites along the highway were designated by Gleba-Lote numbers.

/zcm.

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Table 2

Numbers of Mammals With a Positive Serology\* to *Trypanosoma cruzi* Antigen. Results From 598 Mammals Collected Along the Transamazon Highway at 4 Sites in the Areas of Marabá and Altamira, Pará, Brazil in 1974-1976.

Mammal Group	G 3/5 <sup>a</sup>	G 61 L02 <sup>a</sup>	G 05 L05 <sup>a</sup>	G 29 L03 <sup>a</sup>	Total <sup>a</sup>	
	Number Positive (%)	Number Positive (%)	Number Positive (%)	Number Positive (%)	Number Positive (%)	
	Total	Total	Total	Total	Total	
Marsupials	5 (24)	21	8 (14)	57	10 (50)	20
Monkeys	3 (60)	-	-	-	-	2
Edentates	1 (50)	2	-	-	-	-
Rodents	13 (14)	95	56 (37)	152	10 (15)	67
Carnivores	-	-	1 (100)	-	-	-
Total	22 (18)	123	65 (31)	210	20 (23)	87
					23 (13)	178
					130 (22)	598

\* Tests performed with the rapid latex agglutination test.  
<sup>a</sup> Sites along the highway were designated by Gleba-Lote numbers /zcm.

Table 3

Numbers of Mammal Sera Positive to *Toxoplasma gondii* in the Indirect Hemagglutination test.  
 Mammals Were Collected Along the Transamazon Highway in the areas of Marabá and Altamira, Pará,  
 Brazil in 1974-1976.

Mammal Group	G 3/5 <sup>a</sup>	G61 102 <sup>a</sup>	G05 105 <sup>a</sup>	G29 103 <sup>a</sup>	Total	
	Specific Titters	Total Specific Titters	Total Specific Titters	Total Specific Titters	Number Positive*	Total
Marsupials	1:64(1) <sup>b</sup>	21	1:128(2)	56	1:4096(1)	29
Monkeys	0	5	-	-	-	0
Edentates	0	2	-	-	-	0
Rodents	0	122	1:128(1) 1:512(1) 1:256(2)	163	1:64(1) 1:512(1) 1:1024(1)	77
Carnivores	-	-	0	1	-	0
Total	0*	150	3	220	3	106
					0	175
					-	6
					-	651

\* A titer of 1:256 was considered positive.

<sup>a</sup> Sites along the highway were designated by Gleba-Lote numbers.

<sup>b</sup> ( ) Number of sera that reacted at specified titers.

/zcm.

Table 4

Numbers of Mammal Sera Positive\* to Brucella sp. Antigen. Data From Collections of Mammals Captured Along the Transamazon Highway, Pará, Brazil, 1974-1976 and Tabulated by Mammal Group, Location Along the Highway, and Habitat at Capture Site.

Location	Forest		Scrub		Cropland		Totals	
	+ <sup>a</sup>	Total						
Altamira G 3/5								
Marsupials	-	36	-	3	-	6	0	45
Monkeys	-	5	-	-	-	-	0	5
Edentates	-	2	-	-	-	-	0	2
Rodents	-	135	-	41	-	27	0	203
Carnivores	-	1	-	-	-	-	0	1
Ungulates	-	2	-	-	-	-	0	2
Altamira G61 L02								
Marsupials	-	54	-	25	-	10	0	89
Bats	-	-	1	3	-	-	1	3
Rodents	1	149	-	81	-	48	1	278
Carnivores	-	1	-	-	-	-	0	1
Marabá G05 L05								
Marsupials	-	37	-	-	-	-	0	37
Edentates	-	2	-	-	-	-	0	2
Rodents	2	58	-	23	-	5	2	86
Marabá G29 L03								
Marsupials	-	41	-	1	-	10	0	52
Monkeys	-	4	-	-	-	-	0	4
Edentates	-	2	-	1	-	-	0	3
Rodents	5	105	-	31	-	48	5	184
Grand Totals	8	634	1	209	0	154	9	997

\* Results from the rapid slide agglutination test

<sup>a</sup> Sera positive to Brucella spp. antigen.

/zcm.

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Table 5  
Numbers of Mammal Sera Positive to Schistosomiasis Antigen in the Indirect Hemagglutination Test. The Mammals Were Collected Along the Transamazon Highway in the Areas of Marabá and Altamira, Pará, Brazil in 1974-1976.

Mammal Group	Altamira				Marabá				Total
	G 3/5 b	G61 L02 b	G05 L05 b	G29 L03 b	Specific Titters	Specific Titters	Specific Titters	Specific Titters	
Specific Titters	Totals	Specific Titters	Totals	Specific Titters	Totals	Specific Titters	Totals	Specific Titters	
Marsupials	1:8(9)*	17	1:8(16) 1:16(7)	52	1:8(13) 1:16(3) 1:23(3)	29	1:8(12) 1:16(4)	39	0
Monkeys	1:8(1) 1:128(1)	5	-	-	-	-	-	2	0
Edentates	-	2	-	-	-	-	-	-	0
Rodents	1:8(10) 1:16(2) 1:32(3) 1:256*(1)	96	1:8(10) 1:16(6) 1:32(4) 1:64(5) 1:128(3)	149	1:8(5) 1:16(6) 1:32(1) 1:64(1)	71	1:8(15) 1:16(6) 1:32(3)	132	1 <sup>a</sup>
Carnivores	-	-	-	1	-	-	-	-	0
Total	1*	120	0	202	0	100	0	173	1 <sup>a</sup>
									595

\* ( ) Number of sera that reacted at specified titers.

<sup>a</sup> A titer of 1:256 was considered positive

b Sites along the highway were designated by Gleba-Lote numbers.  
/zcm.

Sera from 945 mammals collected in the forest, secondary scrub, and cropland were tested for the presence of antibodies to leptospirosis by the rapid slide agglutination test, and 70 were found positive (Table 6). The rodents produced antibodies in 50 of the 711 (7%) sera tested and marsupials in 17 of 215 (7.9%). Nectomys squamipes, a rodent which is normally found in or near water, had the highest percentage of antibodies. Of the 40 N. squamipes tested, 14 (35%) were positive. Sera from 3 rodents collected in houses were also tested, and 1 rodent collected in the house at gleba 29 lote 03 was found to have antibodies.

Sera from 819 mammals were tested for antibodies against the following viruses: Araguarí, EEE, WEE, Mayaro, Mucambo, yellow fever, Ilhéus, St. Louis, Sp H34675, Marituba, Caraparú, Catú, Guama, Icoaraci, Itaporanga, Oropouche, Utinga, Guara, and Tacaima (Table 7). One hundred fifteen mammals had antibodies to 1 or more of these viruses. The serum from 1 rodent (Oxymycterus sp.) was seropositive to 6 viruses: Marituba, Caraparú, Catú, Guama and Tacaima. The serum from another rodent (Neacomys spinosus) had antibodies to Caraparú, Itaporanga, Guama, and Icoaraci. The remaining mammal sera were positive to 1 or 2 viruses. None of the mammal sera contained antibodies to the following 7 viruses: Araguari, EEE, yellow fever, Mucambo, Oropouche, Utinga or Sp H34675. Antibody rates were highest to Caraparú, Guama, Icoaraci, and Itaporanga viruses. Caraparú and Guama virus infections were most common in gleba 61 lote 02, while Icoaraci and Itaporanga were common at all 4 collecting sites. Proechimys guyannensis and P. longicaudatus tended to have a higher percentage of sera containing antibodies to the viruses tested, 24% and 27% respectively, than the other mammalian species (Table 8). Oryzomys capito and Oxymycterus sp. had a very low percentage of sera containing antibodies, 1% and 3% respectively.

A panel of sera from 291 mammals was sent to the Division of Hazardous Microorganisms, WRAIR, to be tested for antibody to plague, tularemia and rickettsia. No plague antibodies were found using the HI test. The test for opsonizing antibodies indicated 1 rodent (Proechimys) sera out of the 264 tested was positive for tularemia. The results of screening 257 sera for rickettsial antibodies are presented in Table 9.

Blood specimens from 2,032 mammals were examined microscopically for presence of anthrax, microfilariae, trypanosomes, and malaria (Table 10). Microfilariae were found in the blood of 7 marsupials (5 Philander opossum, 1 Marmosa cinerea, and 1 Caluromys philander). One Philander opossum, 1 Caluromys philander and 1 Oryzomys, a rodent, had trypanosomes in their

Table 6  
 Numbers of Mammal Sera Positive\* to Leptospiral Antigen. Data  
 From Collections of Mammals Captured Along the Transamazon  
 Highway, Pará, Brazil, 1974-1976 and Tabulated by Mammal Group,  
 Location Along the Highway and Habitat at Capture Site.

Mammal Groups by Location	Test Results by Location, Mammal Group and Habitat							
	Forest		Scrub		Cropland		Totals	
	+ <sup>a</sup>	Total	+ <sup>a</sup>	Total	+ <sup>a</sup>	Total	+ <sup>a</sup>	Total
Altamira G 3/5								
Marsupials	6	40	1	3	-	6	7	49
Monkeys	-	5	-	-	-	-	0	5
Edentates	-	2	-	-	-	-	0	2
Rodents	8	127	4	40	6	29	18	196
Carnivores	-	1	-	-	-	-	0	1
Ungulates	1	2	-	-	-	-	1	2
Altamira G61 L02								
Marsupials	5	60	-	12	1	13	6	85
Rodents	3	125	5	97	2	44	10	266
Carnivores	-	1	-	-	-	-	0	1
Marabá G05 L05								
Marsupials	3	39	-	-	-	-	3	39
Edentates	-	2	-	-	-	-	0	2
Rodents	4	61	3	23	2	5	9	89
Marabá G29 L03								
Marsupials	1	41	-	1	-	-	1	42
Monkeys	1	4	-	-	-	-	1	4
Edentates	-	1	1	1	-	-	1	2
Rodents	9	107	2	29	2	24	13	160
Total	41	618	16	206	13	121	70	945

\* Results from the rapid slide agglutination test.

<sup>a</sup> Sera positive to leptospiral antigen.

/zcm.

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Table 7

Results of Hemagglutination Inhibition Tests of 819 Mammalian Sera for Antibody to Various Arboviruses. Sera Were Collected at 2 Sites Near Altamira and 2 Sites Near Marabá, Pará, Brazil in 1974-1976\*.

Virus	G <sup>a</sup> 3/5		G <sup>a</sup> 61 L02		G <sup>a</sup> 05 L05		G <sup>a</sup> 29 L03		Total	
	+ <sup>b</sup>	Total	+ <sup>b</sup>	Total	+ <sup>b</sup>	Total	+ <sup>b</sup>	Total	+ <sup>b</sup>	Total
Araguari	-	207	-	291	-	122	-	199	0	819
EEE	-	207	-	291	-	122	-	199	0	819
WEE	1	207	-	291	-	122	-	199	1	819
Mayaro	2	207	1	291	-	122	3	199	6	819
Mucambo	-	207	-	291	-	122	-	199	0	819
yellow fever	-	207	-	291	-	122	-	199	0	819
Ilheus	3	207	1	291	-	122	-	199	4	819
St. Louis	1	207	1	291	-	122	4	199	6	819
Sp H 34675	-	207	-	291	-	122	-	199	0	819
Marituba	-	207	6	291	-	122	1	199	7	819
Caraparú	-	207	13	291	-	122	-	199	14	819
Catú	-	207	1	291	-	122	-	199	1	819
Guamá	4	207	16	291	3	122	2	199	25	819
Icoaraci	10	207	19	291	21	122	21	199	61	819
Itaporanga	8	207	9	291	18	122	18	199	43	819
Oropouche	-	207	-	291	-	122	-	199	0	819
Utinga	-	207	-	291	-	122	-	199	0	819
Guaroa	-	207	1	291	-	122	-	199	2	819
Tacaiuma	-	207	1	291	-	122	-	199	1	819
Total	29	207	69	291	23	122	50	199		
% Sera Positive	14%		24%		19%		25%			

\* Several sera contained antibodies against more than one virus.

<sup>a</sup> Sites along the highway were designated by Gleba-Lote numbers.

<sup>b</sup> Numbers of positive sera.

/zcm.

Table 8

Results of the HI Test for the Presence of Antibodies to Araguari, EEE, WEE, Mayaro, Mucambo, yellow fever, Ilhéus, St. Louis, Sp H 34675, Marituba, Caraparú, Catú, Guamá, Icoaraci, Itaporanga, Oropouche, Utinga, Guaroa and Tacaima Viruses. Mammals Were Collected Along the Transamazon Highway, Pará, Brazil in 1974-76.

Species	G <sup>a</sup> 3/5		G <sup>a</sup> 61 L02		G <sup>a</sup> 05 L05		G <sup>a</sup> 29 L03		Totals	
	+b	Total	+b	Total	+b	Total	+b	Total	+b	Total
<u>Caluromys philander</u>	-	4	-	1	-	1	-	1	0	7
<u>Monodelphis brevicaudata</u>	-	4	1	18	-	1	1	1	1	24
<u>Marmosa cinerea</u>	-	2	-	1	-	4	-	2	0	9
<u>M. murina</u>	-	1	-	-	-	-	-	1	0	2
<u>Philander opossum</u>	2	4	4	25	-	2	2	27	8	58
<u>Metachirus nudicaudatus</u>	1	3	1	8	-	1	1	4	3	16
<u>Didelphis marsupialis</u>	1	16	-	21	2	28	2	6	5	71
<u>Callicebus torquatus</u>	1	1	-	-	-	-	-	-	1	1
<u>Alouatta belzebul</u>	1	2	-	-	-	-	1	2	2	4
<u>Callithrix argentata</u>	-	1	-	-	-	-	-	-	0	1
<u>Saguinus tamarin</u>	-	-	-	-	-	-	1	2	1	2
<u>Dasyurus sp.</u>	2	2	-	-	-	2	-	2	2	6

Table 8. Continued

<u>Oryzomys</u>											
<u>bicolor</u>	-	-	-	-	-	2	-	-	0	2	
<u>O. capito</u>	-	32	-	8	1	18	-	29	1	87	
<u>O. concolor</u>	-	3	1	2	-	-	-	5	1	10	
<u>O. macconnelli</u>	-	3	2	3	-	1	-	2	2	9	
<u>O. fulvescens</u>	-	-	-	-	-	-	1	2	1	2	
<u>O. (Oecomys) sp?</u>	-	1	-	-	-	-	-	-	0	1	
<u>O. sp.?</u>	-	-	-	2	-	-	-	-	0	2	
<u>Neacomys</u>											
<u>guianae</u>	-	-	-	-	-	2	-	1	0	3	
<u>N. spinosus</u>	-	-	1	2	-	-	-	-	1	2	
<u>Nectomys</u>											
<u>squamipes</u>	-	6	2	9	-	-	-	23	2	38	
<u>Zygodontomys</u>											
<u>lasiurus</u>	-	-	-	-	2	25	2	18	4	43	
<u>Oxymycterus</u> sp.	-	37	3	54	-	-	-	1	3	92	
<u>Holochilus</u>											
<u>brasiliensis</u>	-	-	-	-	-	-	-	2	0	2	
<u>Rattus</u> <u>rattus</u>	-	1	-	4	-	1	-	13	0	19	
<u>Agouti</u> <u>paca</u>	-	-	1	2	-	-	-	1	1	3	
<u>Dasyprocta</u>	3	5	-	1	-	-	-	-	3	6	
<u>Proechimys</u>											
<u>guyannensis</u>	1	34	25	82	5	9	2	14	33	139	
<u>P.</u>											
<u>longicaudatus</u>	8	37	6	44	5	25	20	40	39	146	
<u>P. sp.?</u>	-	1	-	-	-	-	-	-	0	1	

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Table 8. Continued.

<u>Mesomys hispidus</u>	-	4	-	3	-	-	-	-	0	1
<u>Nasua nasua</u>	-	1	-	-	-	-	-	-	0	1
<u>Eira barbara</u>	-	-	1	1	-	-	-	-	1	1
<u>Mazama americana</u>	-	2	-	-	-	-	-	-	0	2
Totals	20	207	48	291	15	122	32	199	115	819

<sup>a</sup> Sites along the highway were designated by Gleba-Lote numbers.

<sup>b</sup> Numbers of positive sera.

/zcm.

Table 9

Results of Screening 257 Mammal Sera for Rickettsial Antibody.  
 The Mammals Were Collected Along the Transamazon Highway, Pará,  
 Brazil, 1974-1976.

Description	Number Positive at Indicated Titer				
	1:20	1:40	1:60	1:80	Total
Spotted fever group					
<u>Agouti paca</u>	-	2	-	-	2
<u>Proechimys</u>	3	1	-	2	6
Epidemic typhus group					
<u>Agouti paca</u>	-	1	-	-	1
<u>Proechimys</u>	-	-	1	-	1
Q fever					
<u>Nectomys</u>	1	-	-	-	1
<u>Agouti paca</u>	-	1	-	-	1
<u>Proechimys</u>	-	1	-	-	1
Totals	4	6	1	2	13

/zcm.

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Table 10

Results of Microscopic Examination of Blood Slides From Mammals Collected Along the Transamazon Highway, Pará, Brazil, 1974-1976.

Agent	Altamira				Marabá				Totals	
	G <sup>a</sup> 3/5		G <sup>a</sup> 61 L02		G <sup>a</sup> 05 L05		G <sup>a</sup> 29 L03			
	+ <sup>b</sup>	Total	+ <sup>b</sup>	Total	+ <sup>b</sup>	Total	+ <sup>b</sup>	Total	+ <sup>b</sup>	Total
Anthrax	-	403	-	520	-	445	-	664	-	2,032
Microfilaria	2	403	2	520	-	445	3	664	7	2,032
Trpanasoma	1	403	-	520	-	445	2	664	3	2,032
Malaria	-	403	-	520	-	445	-	664	-	2,032

<sup>a</sup> Sites along the highway were designated by Gleba-Lote numbers.

<sup>b</sup> Positive slides.

/zcm.

blood. No anthrax or malaria infections were found.

The results from the 600 pathological specimens of lung, liver, heart, kidney, spleen, stomach, large intestine, and small intestine were sent to the Department of Veterinary Pathology, WRAIR, to be examined. Resultant data are not available at this time. During the surveillance program, a total of 2,328 cases for pathological examination were preserved in 10% formalin.

COMMENTS: Few mammal sera or blood slides produced pathogenic organisms other than the native viruses which were commonly found. The colonist population is still relatively low in numbers and scattered along the Transamazon Highway. Livestock are presently being introduced, but are not common. As more colonists and laborers from other areas of Brazil migrate to the Transamazon area, and the population density becomes greater, one would expect to find a higher index of introduced diseases in the sylvatic mammal population. The introduction of more livestock will also have its effect, as Brucellosis, for example, is common in most of South America.

#### Habitat Preference and Relative Abundance.

During the mammal surveillance program a total of 3,068 mammals were collected. Most were collected by trapping, 3,008, while 49 were shot and 11 bats were collected with mist nets (Table 11). More than 50% of the animals were collected in the forest. Of the 47 species collected, 41 species were represented in collections conducted in the forest, 29 in the secondary scrub and 20 in the cropland. This is to be expected, as the forest habitat is much more diverse than either the secondary scrub or cropland. The marsupials, with the exception of Monodelphis, tended to prefer the forest over the disturbed areas. Oryzomys capito, O. macconnelli, Neacomys guianae, N. spinosus, Proechimys guyannensis, and P. longicaudatus definitely preferred the forest over the disturbed habitats, while population densities of Oryzomys fulvescens, Zygodontomys lasiurus and Oxymycterus sp. increased when the forest was cleared for cropland. Mesomys hispidus, and infrequently collected arboreal rodent, clearly preferred the forest habitat.

A total of 45,661 trap nights in the 4 collecting sites produced 3,008 mammals, or 15.2 trap nights per animal collected. The most common species in all sites were Oryzomys capito, Proechimys guyannensis, and P. longicaudatus (Table 12). A few species such as Oryzomys fulvescens, Neacomys guianae, N. spinosus, Zygodontomys lasiurus, and Oxymycterus sp. were common in 1 or 2 sites, but rare or not collected in others. Rattus rattus was very common

Table 11  
Species and Numbers of Mammals Captured Along the Transamazon Highway, Para, Brazil, 1974-1976.  
Numbers Collected are Listed by Habitat at Capture Site.

Species	Collection Method*	Habitat Type			Total
		Forest	Secondary Scrub	Cropland	
<b>Marsupialia</b>					
<i>Caluromys philander</i>	T	11	-	1	12
<i>Monodelphis brevicaudata</i>	T	9	3	32	72
<i>M. b. (touan)?</i>	T	3	-	2	8
<i>Marmosa cinerea</i>	T	31	-	-	31
<i>M. murina</i>	T	10	1	1	12
<i>M. parvidens</i>	T	7	-	-	7
<i>Philander opossum</i>	T	78	-	-	78
<i>Metachirus nudicaudatus</i>	T	23	1	-	24
<i>Didelphis marsupialis</i>	T	81	2	-	83
 <b>Chiroptera</b>					
<i>Peroteryx macrotis</i>	N	-	1	-	1
<i>Pteronotus parnellii</i>	N	-	1	-	1
<i>Carollia subrufa</i>	T-N	3	3	-	6
<i>Sturnira lilium</i>	N	-	2	-	2
<i>S. tildae</i>	N	-	3	-	3
<i>Ectophylla macconnelli</i>	N	-	1	-	1
<i>Artibeus jamaicensis</i>	T	-	-	-	1

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Table 11. Continued

Primates								
<u>Callicebus torquatus</u>	5	-	-	-	-	5	4	
<u>Alouatta belzebul</u>	4	-	-	-	-	1		
<u>Cebus apella</u>	1	-	-	-	-	2		
<u>Callithrix argentata</u>	2	-	-	-	-	3		
<u>Saguinus tamarin</u>	3	-	-	-	-			
Edentate								
<u>Dasyurus</u>	6	1	-	-	7			
Lagomorpha								
<u>Sylvilagus brasiliensis</u>	5	-	-	1	1			
Rodentia								
<u>Sciurus</u>	T-S	1	1				2	
<u>Oryzomys bicolor</u>	T	18	1				21	
<u>O. capito</u>	T	485	27				560	
<u>O. concitor</u>	T	24	10				42	
<u>O. macconnelli</u>	T	90	4				95	
<u>O. fulvescens</u>	T	34	249				376	
<u>O. (Oecomys) sp?</u>	T	1	2				3	
	T	17	8				35	

Table 11. Continued

Rodentia							
<u>Neacomys guianae</u>	T		5		2		76
<u>N. spinosus</u>	T	44	8		29		81
<u>Nectomys squamipes</u>	T	34	12		14		60
<u>Zygodontomys lasiurus</u>	T	14	328		118		460
<u>Oxymycterus</u> sp.	T	2	116		80		198
<u>Holocephalus brasiliensis</u>	T	2	4	-	14		6
<u>Rattus rattus</u>	T	11	-	-	-		25
<u>Agouti pacá</u>	S	7	-	-	-		7
<u>Dasyprocta</u>	S	11	-	-	-		11
<u>Proechimys guyannensis</u>	T	171	21		43		235
<u>P. longicaudatus</u>	T	308	23		17		348
<u>P. sp?</u>	T	7	1		1		9
<u>Mesomys hispidus</u>	T	10	-		-		10
Carnivora							
<u>Nasua nasua</u>	S		3	-	-		3
<u>Eira barbara</u>	S		1	-	-		1
Artiodactyla							
<u>Mazama americana</u>	S		3	-	-		3
Totals			1,645	870	517		3,032

\* Specimens were collected either by live traps (T), mist nets (N) or shooting (S). /cm.

Table 12

Mammals Collected per 10,000 Trap Nights at 4 Trapping Sites  
Along the Transamazon Highway, Pará, Brazil, 1974-1976

Species	Altamira		Marabá	
	G 3/5*	G61 L02*	G05 L05*	G29 L03*
<b>Marsupialia</b>				
<u>Caluromys philander</u>	4.24	3.07	1.62	1.69
<u>Monodelphis</u> <u>brevicaudata</u>	19.62	42.96	2.43	3.38
<u>M. b. (touan)?</u>	-	-	2.43	4.22
<u>Marmosa cinerea</u>	6.82	4.09	10.54	5.07
<u>M. murina</u>	1.70	3.07	1.62	4.22
<u>M. parvidens</u>	0.85	-	4.87	-
<u>Philander opossum</u>	5.97	35.80	1.62	28.73
<u>Metachirus</u> <u>nudicaudatus</u>	3.41	8.18	3.24	6.76
<u>Didelphis</u> <u>marsupialis</u>	17.91	25.57	21.90	8.45
<b>Rodentia</b>				
<u>Sciurus</u>	-	-	0.81	-
<u>Oryzomys bicolor</u>	2.56	1.02	7.30	6.76
<u>O. capito</u>	106.62	39.89	123.31	206.18
<u>O. concolor</u>	10.24	15.34	1.62	10.98
<u>O. macconnelli</u>	3.41	9.20	10.54	58.31
<u>O. fulvescens</u>	-	-	35.69	280.55
<u>O. (Oecomys) sp?</u>	0.85	1.02	-	0.84
<u>O. sp. ?</u>	0.85	6.14	4.87	19.44
<u>Neacomys guianae</u>	-	-	30.02	32.96
<u>N. spinosus</u>	24.74	52.17	-	-
<u>Nectomys squamipes</u>	5.97	11.25	0.81	34.64
<u>Zygodontomys</u> <u>lasiurus</u>	-	-	262.03	115.77
<u>Oxymycterus</u> sp.	62.26	120.70	3.24	2.54
<u>Holochilus</u> <u>brasiliensis</u>	-	-	-	5.07

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Table 12. Continued

Rodentia				
<u>Rattus rattus</u>	0.85	3.87	0.81	15.21
<u>Proechimys guyannensis</u>	50.32	132.98	9.73	28.73
<u>P. longicaudatus</u>	52.03	63.42	67.33	119.99
<u>P. sp. ?</u>	1.70	-	1.62	4.22
<u>Mesomys hispidus</u>	5.12	3.07	-	0.84
Total	388.94	584.08	622.21	1,008.96
Nº of Trap Nights	11,724	9,776	12,327	11,834

\* Locations along the highway are designated by Gleba-Lote numbers.

/zcm.

in colonist's houses, and a few were collected in cropland and forest at distances of 1 km from buildings.

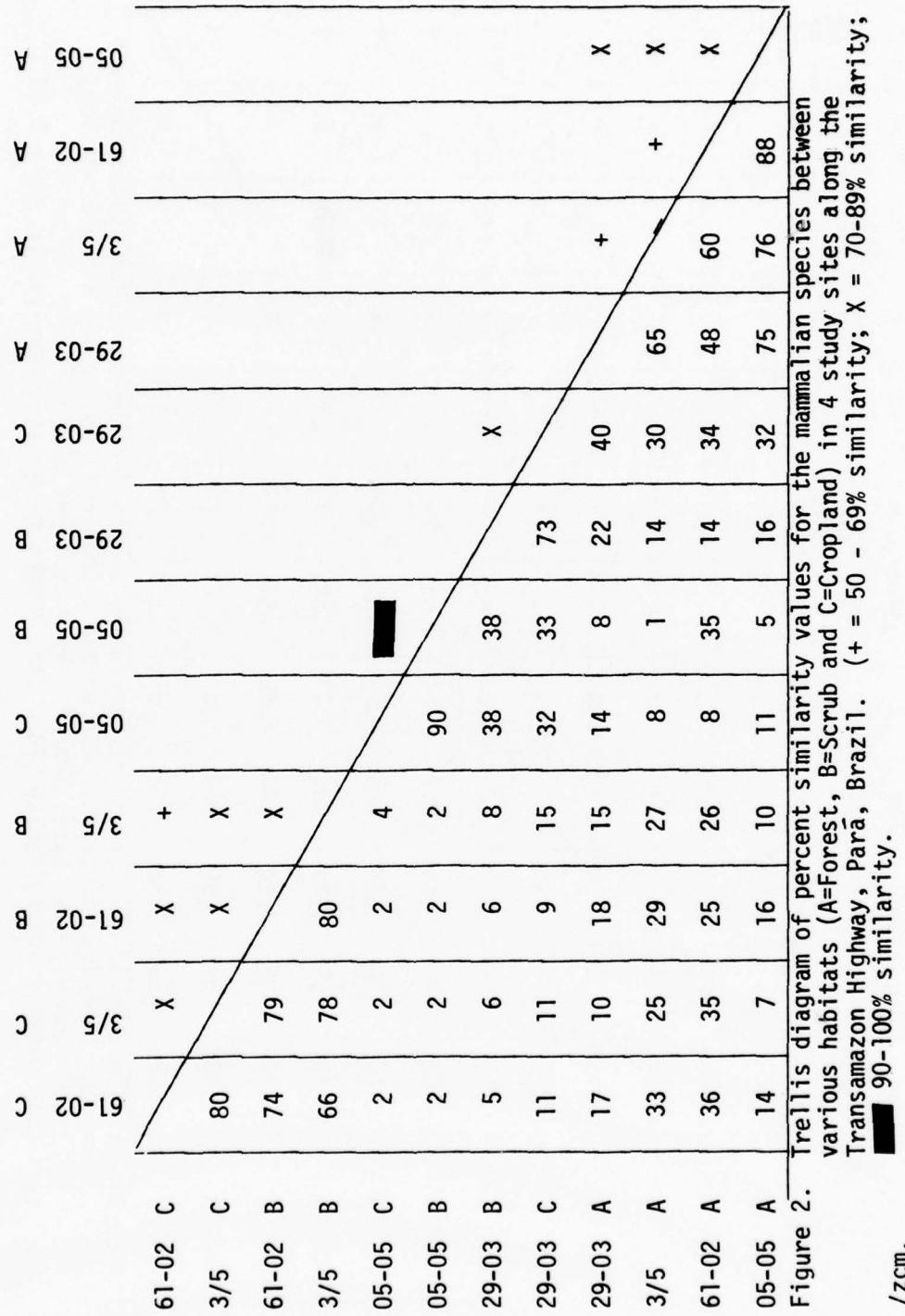
#### Diversity Indices

The faunal composition in forest habitats between the 4 collection sites, evaluated by "percentage similarity" values, was 48% to 88% similar (Figure 2).

Such indices for cropland and secondary scrub habitats between sites in the Altamira area were also high, 66-80%. Identical values between sites in Marabá were very low, although, within site comparisons of cropland and secondary scrub habitats produced high indices for both collection sites in Marabá (Figure 2). There was very little similarity between the cropland and secondary scrub habitats of the Altamira area and the same habitats from the Marabá area.

The rare species indices (quotient of similarity) were generally high for the between and within site comparisons for all habitats (figure 3). The forest mammals were more similar, based on between site comparisons, in species composition than those recorded for the cropland or secondary scrub habitats (disturbed areas). The majority of species that were common in the forest did not adapt and become numerous in cleared areas or in areas of secondary scrub vegetation. The disturbed areas are, therefore, more favorable to another species (or complex of species) becoming dominant. One or more of these species may be introduced from another area.

COMMENT: The practices of land clearing and farming are continuing along the Transamazon Highway and it is expected that the presence and abundance of mammal species will change simultaneously. It seems likely that some vertebrates reservoirs of human disease will be favored by these changes and their population densities will increase. Interesting observations could be made if comparable data were collected from the same areas, 4-5 years in the future.



Trellis diagram of percent similarity values for the mammalian species between various habitats (A=Forest, B=Scrub and C=Crop land) in 4 study sites along the Transamazon Highway, Pará, Brazil. (+ = 50 - 69% similarity; x = 70-89% similarity; ■ = 90-100% similarity.

12cm.

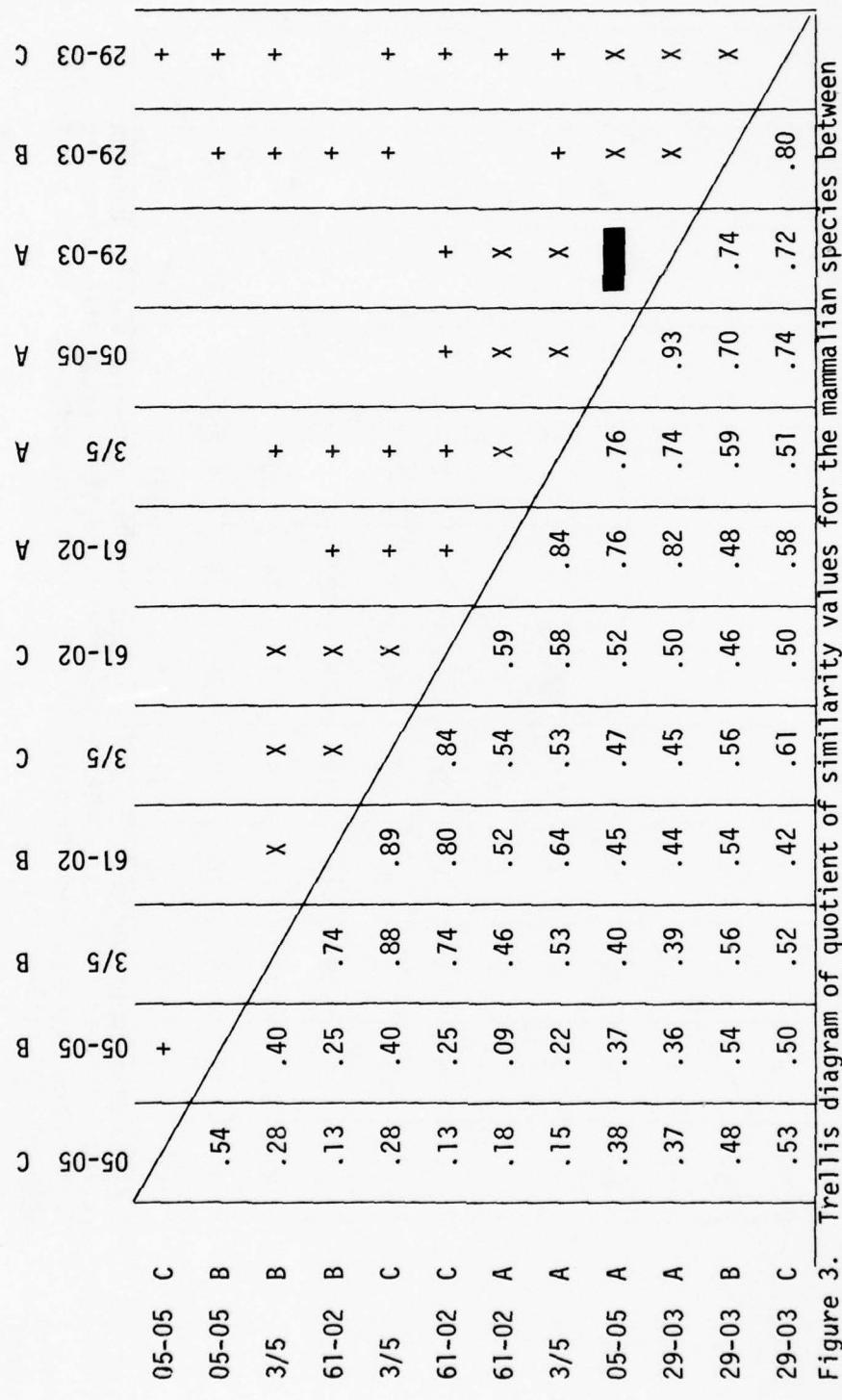


Figure 3. Trellis diagram of quotient of similarity values for the mammalian species between various habitats (A=Forest, B=Scrub and C=Cropland) in 4 study sites along the Transamazon Highway, Pará, Brazil. (+ = Quotient of similarity .50 - .69; X = Quotient of similarity .70 - .89; ■ = Quotient of similarity .90 - 1.00).

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### C. ENTOMOLOGICAL SURVEILLANCE

OBJECTIVE: To document the temporal and spatial diversity and/or similarity of the mosquito fauna along the Transamazon highway, Pará, Brazil.

BACKGROUND: A program of entomological surveillance was conducted at 12 sites along the Transamazon highway from 1974-76. The broad geographic coverage and systematic character of the resultant data was ideal for computing descriptive statistics for the mosquito fauna.

DESCRIPTION: Main emphasis in the schedule of collections was on landing collections (collections of insects landing on man or "man-biting collections"). In addition, collections were routinely conducted with the Shannon traps and Chamberlain light traps. Details of the more general objectives, collecting methods and daily collecting schedule have been presented in the Annual Reports for FY's 1975 and 1976. Descriptions of the 12 collection sites are presented in Tables 1 and 2.

A great volume of entomological data were collected during the surveillance program. It was obvious that species lists accompanied by numbers of specimens would not suffice in attempts to present a succinct description of the spatial and temporal diversity of the mosquito fauna in the study region. Thus, 2 faunal indices were selected to facilitate the analysis and presentation of the descriptive data, *viz.* Sorensens (1948) "Quotient of similarity" and the "percentage of similarity" (Raabe, 1952). The formula for the quotient of similarity (QS) is:

$$QS = \frac{2j}{a+b}$$

where a = the number of species in habitat A,  
b = the number of species in habitat B, and  
j = number of species found in both habitats (Southwood, 1966).

This quotient measures the relative similarity of 2 faunas in terms of species composition. Since there is no emphasis on numbers of specimens per species - this is a qualitative index.

The "percentage of similarity" is more quantitative since it provides a comparison of the numbers of specimens for the various species in 2 samples (or group of samples). Computation of the "percentage of similarity" (%S) is as follows:

Table 1

Description of 12 Study Sites Along the Transamazon Highway in Pará, Brazil. Sites were Routinely Surveyed for Medically Important Insects During 1974-1976.

Collecting Sites	General Site Description				Description of Colonist House			
	Location (Gleba-Lote)	Streams or Rivers Present	Standing Water	Type of House	Relief at Site of House	Distance From Forest Edge	Distance to River or water impoundment	
A	605 L05	stream	1 -1.5 ha	mud walls thatched roof	lower hill slope	ca. 500 m	75 m	
B	G29 L03	stream	1.5-1 ha	wood structure	flat land	750-1000 m	125 m	
C	G38 L06	stream	1 -1.5 ha	thatched	upper slope of hill	85-100 m	150 m	
D	G66 L09	none	.5- .75ha	mud walls thatched roof	lower slope of hill	450-500 m	250 m	

Table 1, Continued

E	G20 L09	river	2 -3	ha	wood structure	lower slope of valley	35 m	500 m
F	G18 L04	stream	rain		wood structure	upper hill summit	140 m	100 m
G	G10 L01	none	none		wood structure	upper hill summit	90 m	-
H	G12 L05	stream	none		wood structure	low slope of hill	75 m	290 m
I	G35 L01	stream	none		mud walls thatched roof	upper hill summit	800 m	375 m
J	G36 L07	stream	rain pools		brick structure	upper hill summit	30 m	55 m
K	G51 L03	stream	stream flood areas		mud walls thatched roof	flat land	550 m	520 m
L	G62 L02	stream	2 -2.5 ha		thatched	low, flat land	25 m	150 m

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/zcm.

Table 2

Description of Forest and Tree Towers at 12 Study Sites Along the Transamazon Highway in Pará, Brazil. Sites were Routinely Surveyed for Medically Important Insects during 1974-1976.

Collecting Site	Forest Description			Description of Tree Tower			
	Height of Emergent Trees	Height of Lower Canopy	Height	Relief at Tower Site	Distance from Forest Edge	Distance to Nearest Stream	
A	20-25+ m	14-18 m	12 m	upper hill, slope	100 m	40-	50 m
B	25-30+ m	16-18 m	12 m	lower hill, slope	250-300 m	750-1000 m	
C	20-25+ m	12-16 m	12 m	upper hill, slope	50 m	350 m	
D	20-25+ m	14-18 m	12 m	lower hill, slope	35- 40 m	-	
E	25-30+ m	10-16 m	10.5m	lower hill, slope	45 m	500 m	
F	25-30+ m	14-18 m	12 m	flat land	75 m	-	
G	20-25+ m	14-18 m	12 m	flat land	90 m	-	

Table 2, Continued

H	20-25+ m	12-16 m	12 m	upper hill, slope	250 m	150 m
I	25-30+ m	14-18 m	14 m	upper hill, slope	100 m	300 m
J	25-30+ m	12-16 m	14.5m	upper hill, slope	30 m	50 m
K	20-25+ m	14-18 m	10 m	flat land	260 m	290 m
L	20-25+ m	12-16 m	10 m	lower hill, slope	20 m	20 m

/zcm.

Habitats A and B with % faunal composition.

Species	a	b	c	d
A	10	50	30	10
B	5	30	20	45

$$\%S = 5 + 30 + 20 + 10 = 65 \text{ (Southwood, 1966)}$$

This value is influenced greatly by the abundant species that are common to both samples (or habitats).

These indices were selected because they provided the required descriptive statistics and were relatively easy to calculate. Their application is defensible since the indices were calculated on samples of comparable large size.

The QS values are useful for indicating the homogeneity in species distribution. The %S values are more relevant to the topics of disease-vector or pest-man associations. Since both indices provide estimates of relative similarity between samples, although with different emphasis, their assessment is necessarily subjective.

The indices (QS and %S) were calculated to compare the mosquito fauna:

1. Between sites along the Transamazon highway for landing collections;

- a) in all habitats and times,
- b) the forest at ground level 1830-1845 hrs.,
- c) in the forest in tree towers, 1400-1500 hrs. and
- d) near the colonists houses, 1855-1910 hrs.

2. Between daily collection times, viz., 0900, 1230, 1600, 1830, 1925 and 2145 hrs., at each site. Collections were conducted in the forest at ground level.

3. Between various types of night collections for each site, as follows:

- a) light trap vs. shannon trap collections,
- b) light trap vs. landing collections, and
- c) shannon trap vs. landing collections.

4. Between light trap collections in forest and cleared areas at each site.

5. Between night landing collections in the forest and cleared areas at each site.

6. Between landing collections conducted at ground level in the forest and in the tree tower at each site.

PROGRESS: The QS values for pooled landing collections by lote were uniformly high (Table 3). These values were calculated on approximately 461 collections per site and included both diurnal and nocturnal collections conducted in cleared areas and in the forest (at ground level and in tree towers). Slightly smaller indices were obtained for collections conducted in the forest at sunset and collections conducted near the colonist houses at sunset (Tables 4 and 5). The between lote QS values for the tree tower collections were also uniformly greater than 0.5 (Table 6). The consistently high indices and lack of distinct trends in site associations by the trellis diagram analyses indicate a homogeneous distribution of mosquito species along the Transamazon highway.

The %S values were lower (with greater range in indices) than the QS values (Tables 7, 8, 9, and 10). There were no great differences in the between lote %S values for 1) total landing collections in all habitats and intervals, 2) landing collections at sunset in the forest and 3) landing collections conducted near the house (Tables 7, 8, and 9). Highest %S were obtained with treetower collections. Collections from sites E and F presented dense populations of Mansonia titillans (Walker) and resulted in a high %S between these sites for all collections.

Landing collections were made in the forest at ground level at 0900, 1230, 1600, 1830, 1925, and 2145 hrs. Comparisons were made between these collection times for each lote. Analysis of the QS and %S values in trellis diagrams revealed associations in the collections by chronological order (Tables 11 and 12). The sunset collection interval was intermediate between day and night collections for both indices, i.e., both day and night active species were collected simultaneously. In addition, the 0900 interval collections were more similar to the 1600 hr collections than to mid-day collections, for both indices. The low values resulting from comparisons between day and night collections reflected separate diurnal and nocturnal populations.

The QS and %S calculations were applied in comparisons of sampling methods and habitats at each of the 12 lotes. The mean QS values

	D	C	I	J	E	L	K	B	A	F	H	G
D	.75											
C	.40	.58										
I	.69	.65	.70									
J	.70	.61	.55	.70								
E	.64	.70	.65	.70	.74							
L	.67	.69	.64	.70	.67	.74						
K	.64	.62	.30	.70	.65	.74	.71					
B	.67	.64	.66	.68	.75	.79	.73	.81				
A	.71	.68	.29	.68	.68	.67	.62	.60	.97			
F	.63	.71	.70	.74	.73	.75	.74	.67	.76	.79		
H	.62	.80	.77	.74	.76	.72	.70	.72	.76	.70	.86	
G												

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Table 3. Trellis Diagram of "Quotient of similarity" Values Between 12 Sites Along the Transamazon Highway, Pará, Brazil for mosquitoes (Culicidae) Collected in Landing Captures, Values Were Calculated From Pooled Data From all Captures Conducted in Various Habitats at Each Site. (+ = QS of 70-79; X = QS of 80-89; █ = QS of 90-99).

/zcm.

	L	I	H	K	J	G	C	E	F	B	D	A
L	.48											
I	.42	.58										
H	.59	.57	.69									
K	.53	.62	.73	.81								
J	.52	.55	.62	.53	.65							
G	.58	.55	.46	.40	.52	.83						
C	.59	.40	.48	.55	.47	.59	.59					
E	.60	.45	.51	.56	.60	.61	.59	.67				
F	.54	.50	.56	.56	.59	.60	.53	.61	.62			
B	.62	.50	.42	.57	.48	.64	.55	.56	.53	.64		
D	.59	.50	.63	.61	.59	.67	.60	.67	.67	.67	.71	
A												+

Table 4. Trellis Diagram of "Quotient of Similarity" Values Between 12 Sites Along the Transamazon Highway in Para, Brazil for Mosquito (Culicidae) Species Collected in the Forest 1830-1845 hrs., 1974-1976 (+ = QS of 70-79; X = QS of 80-89).

/zcm.

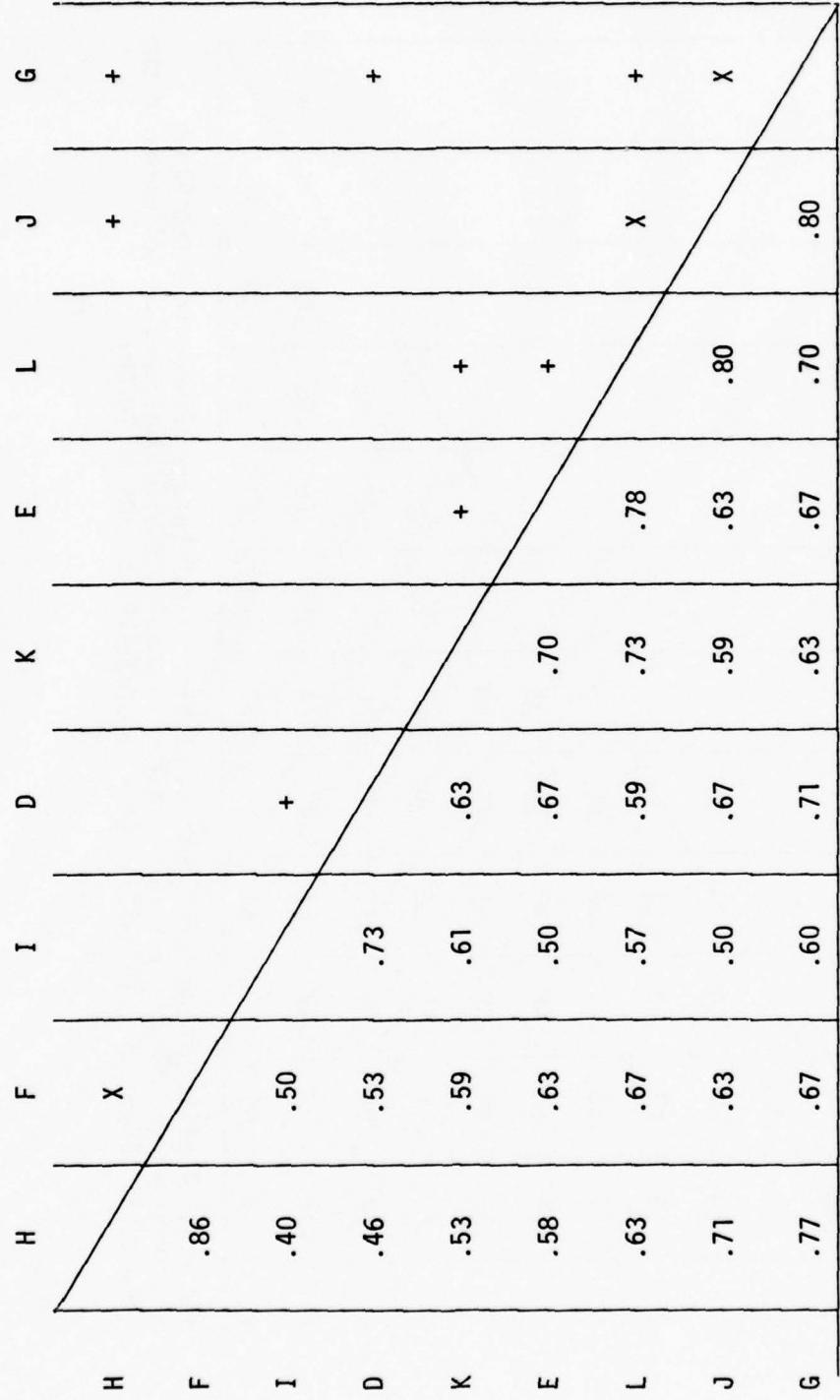


Table 5. Trellis Diagram of "Quotient of Similarity" (QS) Values Between 9 Sites Along the Transamazon Highway in Pará, Brazil for Mosquitoes (Culicidae) Collected Near Colonist Houses in Routine Landing Captures at 1855-1910 hrs., 1974-1976 (+ = QS of 70-79; X = 80-89). /zcm.

	B	I	H	E	G	D	K	A	J	F	C	L
B	.42											
I		.47	.56									
H			.50	.55								
E				.53	.47	.63						
G					.53	.59	.63	.67				
D						.56	.53	.42	.48	.40	.60	
K							.60	.48	.52	.64	.58	.67
A								.58	.55	.58	.52	.70
J									.57	.60	.70	.64
F										.55	.59	.69
C											.63	.60
L												.71

+      X      +      +      +      +      +      .72      +      +      .83      X

Table 6. Trellis Diagram of "Quotient of Similarity" Values Between 12 Sites Along the Transamazon Highway in Pará, Brazil for Mosquitoes (Culicidae) Collected in Tree Towers, 1400-1500 hrs., 1974-1976 (+ = QS of 70-79; X = QS of 80-89).

/zcm.

	E	F	H	B	K	C	I	A	G	D	L	J
	.82											
E	X											
F												
H	.10	.26										
B	.18	.22	.29									
K	.15	.23	.26	.33								
C	.22	.20	.38	.34	.38							
I	.13	.25	.39	.47	.33	.58						
A	.39	.42	.32	.46	.30	.25	.48					
G	.21	.27	.35	.44	.41	.37	.49	.69				
D	.9	.22	.23	.51	.38	.27	.44	.67	.71			
L	.22	.26	.23	.49	.42	.26	.43	.53	.51	.54		
J	.17	.27	.37	.63	.51	.32	.52	.49	.58	.58	.69	

Table 7. Trellis Diagram of "Percentage of Similarity" (%S) Values Between 12 Sites Along the Transamazon Highway, Pará, Brazil for Mosquitoes (Culicidae) Collected in Landing Captures. Values Were Calculated From Pooled Data From all Captures Conducted in Various Habitats at Each Site from 1974-1976 (+ = %S of 51-75; X = %S of 76-100)

/zcm.

	K	H	C	L	E	F	G	A	D	B	I	J
K	34											
H		19	35									
C				29								
L					10							
E						15						
F	24						38					
G	36							50				
A	19	12							+			
D	27	25								+		
B	31	29									+	
I	23	30										+
J	36	46										+

Table 8. Trellis Diagram of "Percentage of Similarity" (%S) Values Between 12 Sites Along the Transamazon Highway, Pará, Brazil for Mosquitoes (Culicidae) Collected in Landing Captures in the Forest at 1830-1845 hrs., 1974-1976 (+ = %S 51-75; X = %S of 76-100).

/zcm.

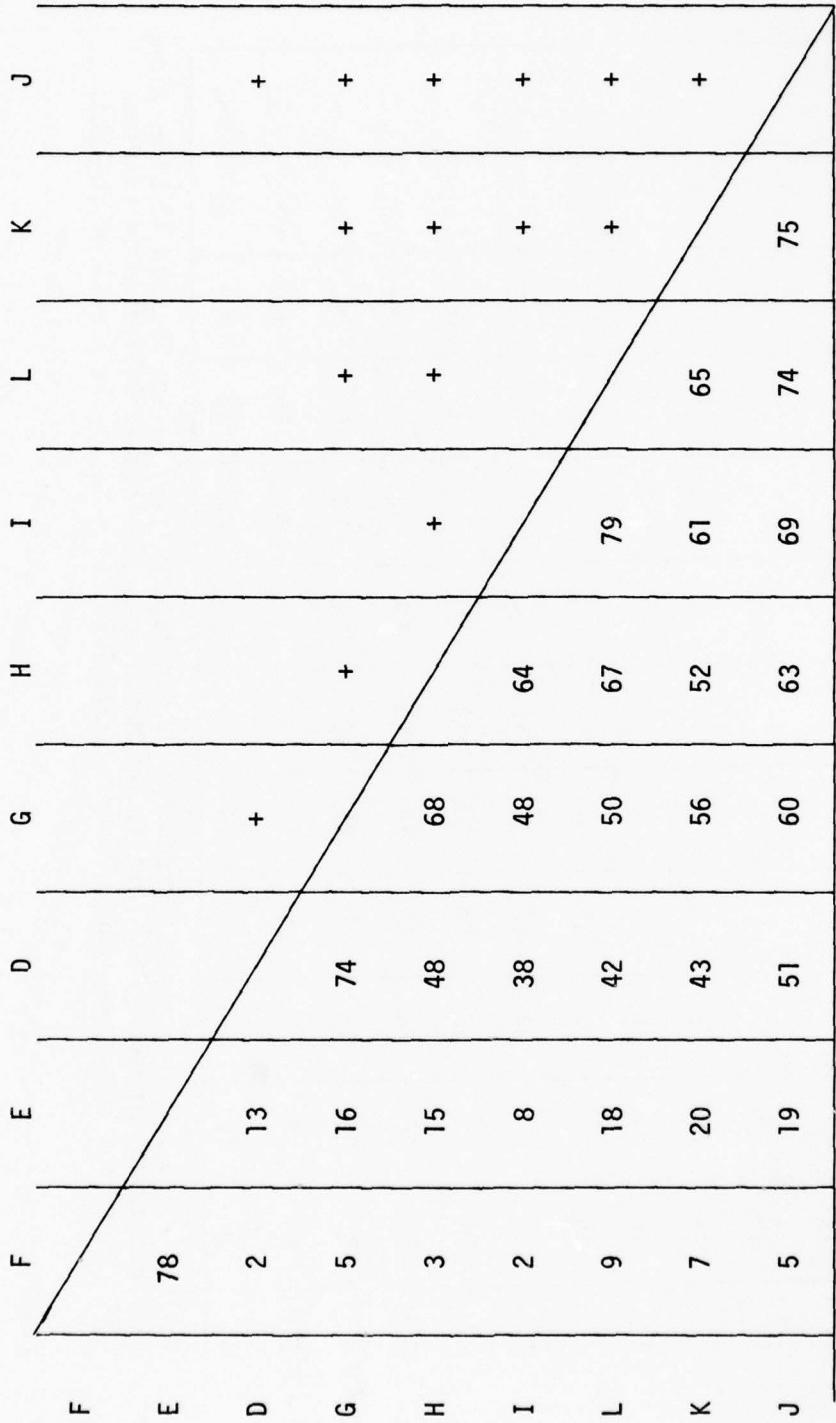


Table 9. Trellis Diagram of "Percentage of Similarity" (%S) Values Between 9 Sites Along the Transamazon Highway, Pará, Brazil for Mosquitoes (Culicidae) Collected in Landing Captures Near Colonist Houses 1855-1900, 1974-1976 (+ = %S of 51-75; X = %S 76-100). /zcm.

	D	C	B	J	K	A	I	G	H	L	F	E
E											+	51
F											+	
L	7	33										
H	10	42	47									
G	9	45	37	59								
I	9	44	36	46	74							
A	21	59	36	34	69	78						
K	9	50	40	47	57	68	74					
J	12	43	43	48	55	56	61	63				
B	7	32	30	40	39	51	53	57				
C	11	44	42	49	49	58	57	65				
D	10	38	50	45	53	58	60	60				

Table 10. Trellis Diagram of "Percentage of Similarity" (%S) values Between 12 Sites Along the Transamazon Highway, Pará, Brazil for mosquitoes (Culicidae) Collected in Landing Captures in Tree Towers 1400-1500 hrs., 1974-1976 (+ = %S of 51-75; X = 76-100).

/zcm.

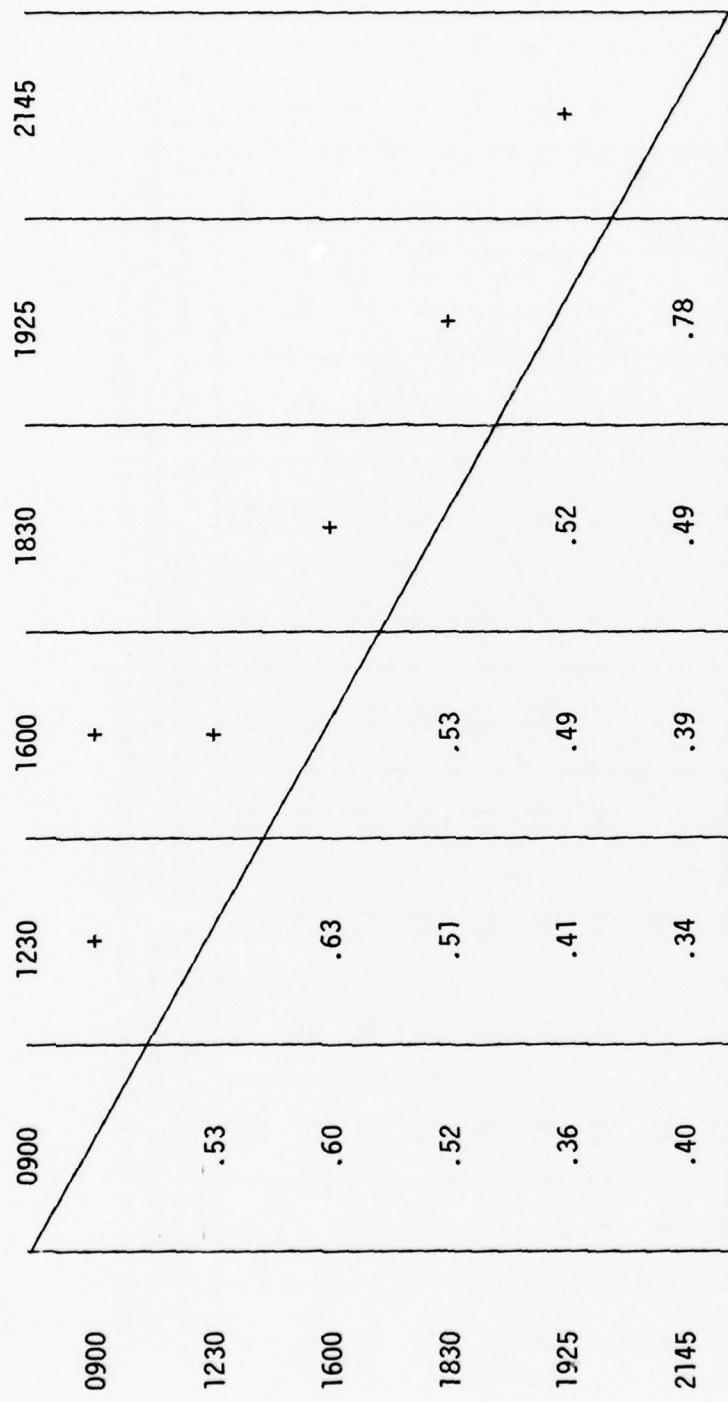


Table 11. Trellis Diagram of Mean "Quotient of Similarity" (QS) Values Between Collection Intervals, by Time of day, From 12 Sites Along the Transamazon Highway, Pará, Brazil. Collections Were Conducted Within the Forest at Each Site During 1974-1976 ( $+ = QS \geq 0.5$ ).

/zcm.

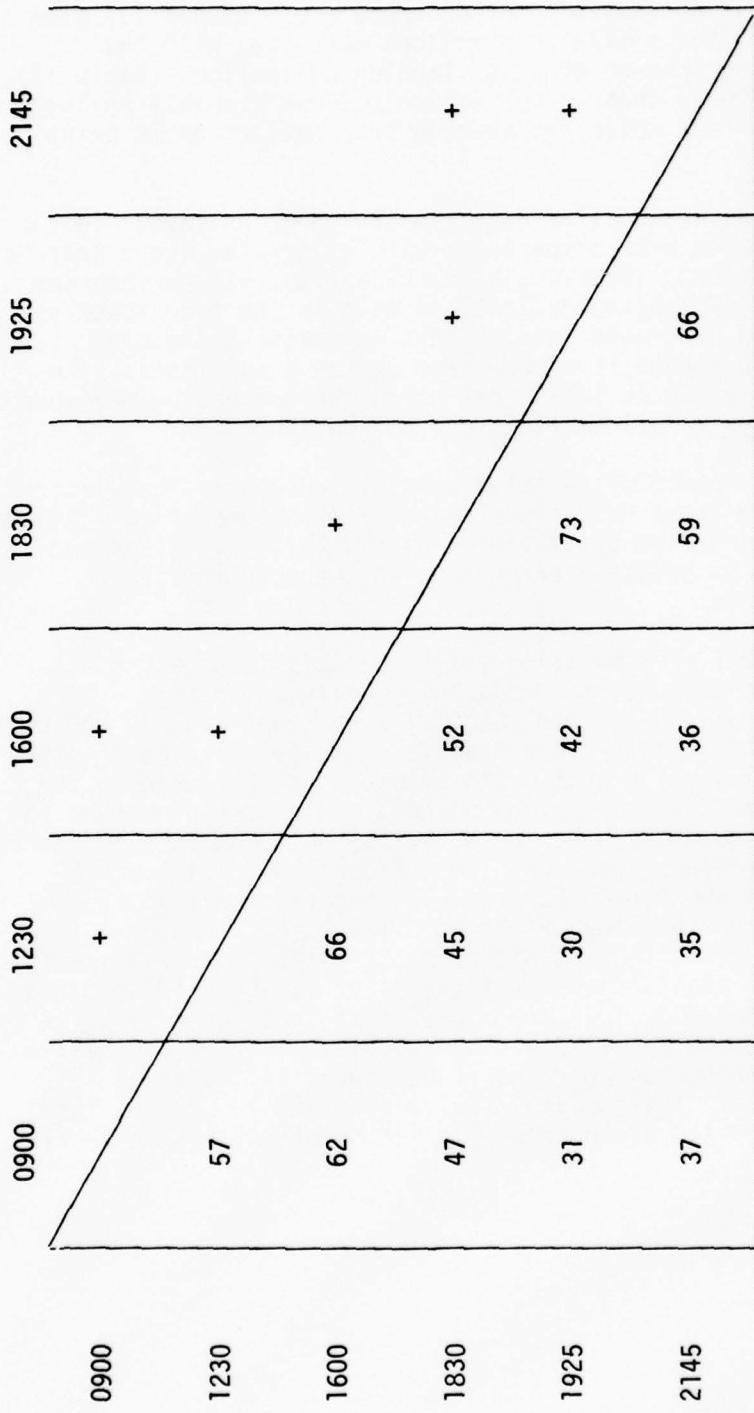


Table 12. Trellis Diagram of Mean "Percentage of Similarity" (%S) Values Between Collection Intervals, by Time of day, From 12 Sites Along the Transamazon Highway, Pará, Brazil. Collections Were all Conducted Within the Forest at Each Site From 1974-1976 (+ = %S  $\geq$  50%).

/zcm.

from comparisons of sampling methods were  $\geq 0.5$  (Table 13). The mean %S values from similar comparisons were low, with the exception of the shannon trap vs. landing collections (Table 14). The high %S between these 2 collection methods probably reflects the presence of man while the shannon trap collection is being conducted.

There were no striking differences in the QS or %S values in the forest vs. cleared area comparisons with either the light trap or landing collections. However, both, QS and %S, values were low in comparisons of landing collections made in the tree tower vs. those conducted at ground level. This indicates a striking separateness in ground level and tree canopy populations. The single high %S value at lot E reflected the dominating presence of Ma. titillans in all habitats and collection times.

COMMENT: This report of faunal diversity indices is the first attempt at reducing a voluminous quantity of entomological data to meaningful descriptive statistics. More detailed analyses are being prepared to relate observations on the more medically important species.

The high quotient of similarity values between lotes along the Transamazon indicate considerable homogeneity in species distribution over a large geographical area (approximately 800 km). Thus, with a sufficiently large sample from any selected site the majority of all species that could possibly be collected in the region, by a particular sampling method, would be represented in the sample. Regardless, the low %S values between lotes indicate that local conditions, at each site, strongly determine which species will be abundant. For example, the close proximity of flood plains from the Xingu river at sites E and F produce conditions for dense populations of Ma. titillans, a pest species. Whereas, faunal associations of lotes G, D, L, and J (determined by high %S values, Table 7) are a result of local conditions at each lot producing relatively dense populations of 3 anopheline species, viz. Anopheles nuneztovari Gabaldon, An. oswaldoi (Perryassu) and An. triannulatus (Neiva and Pinto). One of the 3 species is suspected as an exophilic vector of malaria in rural areas.

Table 13

"Quotient of similarity" Values for Mosquitoes Collected in Various Habitats by 3 Collection Methods at 12 Study Sites along the Transamazon Highway, Pará, Brazil from 1974-1976.

Types of Collections	Collection Sites											
	A	B	C	D	E	F	G	H	I	J	K	L
Forest at ground level, night collections												
Light trap vs Shannon trap	62	50	35	50	71	40	35	26	44	79	63	72
Light trap vs landing captures	53	59	48	54	63	65	57	41	42	40	61	15
Shannon trap vs landing captures	65	67	33	33	63	56	76	82	55	50	63	67
Forest vs cleared areas, night collections												
Light trap	63	72	60	56	84	55	35	56	40	77	67	65
Landing captures*	-	-	-	89	44	53	63	30	53	37	55	52
Forest, day time landing collections												
Tree tower vs ground level	44	41	21	40	42	47	41	42	20	47	36	20
* Insufficient sample size for sites A, B and C. /zcm.												36

Table 14

**"Percentage Similarity" Values for Mosquitoes Collected in Various Habitats by 3 Collecting Methods at 12 Study Sites along the Transamazon Highway, Pará, Brazil from 1974-1976.**

Types of Collections	Collection Sites							I	J	K	L	$\bar{X}$	
	A	B	C	D	E	F	G						
Forest at ground level, night collections													
Light trap vs Shannon trap	60	43	45	18	43	40	32	35	42	27	22	40	37
Light trap vs landing captures	27	27	36	61	37	27	48	28	26	18	69	13	35
Shannon trap vs landing captures	50	54	31	90	60	62	54	42	70	56	75	50	58
Forest vs cleared areas, night collections													
Light trap	35	44	52	28	81	44	48	81	75	73	34	66	53
Landing captures*	-	-	-	48	74	38	62	9	82	36	37	35	47
Forest, day time landing collections													
Tree tower vs ground level	19	22	6	6	47	26	14	7	8	21	25	10	18

## II. VECTORS AND NATURAL RESERVOIRS OF OROPOUCHE VIRUS IN THE AMAZON REGION

### A. FIELD SURVEILLANCE FOR VERTEBRATE RESERVOIRS OF OROPOUCHE VIRUS

OBJECTIVE: Objectives of this program are to determine which species of mammals and/or birds may serve as a reservoir to Oropouche virus in a sylvatic environment, and to elucidate their role in the disease cycle. Habitat preferences, breeding cycles, and annual population fluctuations of incriminated species will also be documented.

BACKGROUND: Oropouche virus was isolated from a human in Trinidad in 1955. It was discovered in Brazil by members of the Evandro Chagas Institute in 1960. Since 1960, epidemics have been documented in Belém, Bragança, Itupiranga, Santarém, Mojui dos Campos and Belterra, Pará, Brazil. Although antibodies to Oropouche virus have been found in sera of various species of mammals and birds, the only recorded isolation from a non-human vertebrate was from a three-toed sloth (Bradypus tridactylus) collected 150 km southeast of Belém. These results suggest that free-living vertebrates play a role in the natural maintenance of Oropouche virus.

DESCRIPTION: Site Description. A program of systematic collections of birds and mammals was begun in a study area located 44 km south and 40 km west of Santarém, Pará at the Curuá Una Hydroelectric Plant (latitude 2°50' south and longitude 54°22' west). Animals are collected on both sides of the Curuá Una river which flows from west to east and is approximately 100 m wide. The general topography of the area ranges from flatland to steep slopes. The river is 47 m above sea level and the hills rise to approximately 100 m. The forest is classified as tropical semi-evergreen seasonal forest (Beard, 1944). The soil along the river is sandy, and ranges from a reddish-brown sandy clay to clay-sand. The A<sub>1</sub> horizon (humus layer) is 1 to 2 cm thick and contains many fine hair-like roots. The A<sub>2</sub> horizon (litter layer) is 4 to 7 cm in thickness, and is composed of fallen leaves and twigs. Moss cover on fallen trees is also common. A shrub layer of thin woody plants, 0.5-3 m tall, is present, but a machete is not required for walking in the forest. Small trees and palms, to 10 m in height, make up an open canopy. Larger trees, from 15 to 20 m tall, are common, and emergents, to 30 m in height, are scattered throughout. Epiphytes are not common, but small vines and lianas are plentiful. Some of the smaller trees have stilt roots, and buttressing is evident on a few of the emergents.

**Collecting Methods.** The National live traps (150 x 150 x 485 mm) and Rinker live traps (80 x 80 x 255 mm) are set out at 10 m intervals along established trails. The traps are left in place until trapping success begins to diminish, usually within 2 to 4 weeks. The Rinker traps were also placed in trees to collect arboreal rodents and marsupials.

The mammal traps are baited with corn and bananas, and checked early in the morning to reduce the number of mammals dying. Larger animals, monkeys, anteaters, etc., are hunted. Animals are hunted both during the night and during the day.

Mist nets, 12 m long and 2.6 m in height are set across natural flyways such as narrow roads, edges of clearings, etc., to capture bats. The mist nets are also set in continuous lines cut through the forest to capture birds. Mist nets set out to capture bats are usually tended from dusk until 2200 hrs. Nets are not set out during the periods of the month when the moon is bright. The mist nets for capturing birds are tended from before dawn until 1100 hrs.

**Methods of Processing Specimens.** The captured vertebrates are transferred to cloth bags and taken to the field laboratory for processing. At the field laboratory each animal is given a collection number which corresponds to the date and area (example: S-010677-01, which is decoded as S-Santarém, 010677 - 1 June 1977, 01 - first specimen processed on this date). Endo-and Ectoparasites collected from each animal are labelled with the same number. Each animal is exsanguinated, 1 cc of whole blood is preserved in liquid nitrogen for virus isolation attempts, a thick and a thin smear is prepared for blood parasite examination, and the remainder is centrifuged and the serum is preserved in liquid nitrogen. After the animal is exsanguinated, it is placed in a paper bag with chloroform, and the ectoparasites are removed. A representative sample is preserved in alcohol for later identification, and the remainder placed in liquid nitrogen to be examined for pathogens. Standard mammal measurements (total length, tail length, hind foot length, ear length, and weight) are recorded from each animal. Organs are removed and preserved, both in liquid nitrogen for isolation attempts, and in 10% formalin for pathological studies. Endoparasites are also preserved in 10% formalin. Preserved specimens are sent to the base laboratory in Belém for processing. Each mammal specimen is preserved either as skin and skull, skull only, or in formalin and shipped to Belém for tentative identification, and later to taxonomists specializing in South American animals to confirm the identification. All the information is recorded on field forms which are described in the 1975 Annual Report.

Virus Serology. Mammal sera are tested, in the Belém laboratory, for antibodies to Araguari, EEE, WEE, Mayaro, Mucambo, yellow fever, Ilheus, St. Louis, Sp H34675, Marituba, Caraparu, Catú, Guama, Icoaraci, Itaporanga, Oropouche, Utinga, Guaroa and Tocaiuma viruses with the HI test.

PROGRESS: The program of mammal trapping, hunting, and mist netting was initiated at the Curuá Una collection site 15 June 1977. During the first 3 months of the collecting program, a total of 458 mammals were collected (Tables 1, 2, and 3). The most common mammals trapped were Proechimys guyannensis and P. longicaudatus; Carolla brevicaudata was the most commonly mist netted bat, followed by Sturnira lilium. Hunting was not very productive in the Curuá Una area, as only 9 specimens were collected. A bird collecting program was initiated on 12 September 1977.

Serum of a Monkey, Callicebus torquatus, contained antibodies to Oropouche virus (titer 1:20), as well as to WEE, Mayaro, Mucambo, and Utinga viruses. The sera of 25 of 29 (86%) spiny rats, Proechimys guyannensis and P. longicaudatus contained antibody to at least one of the viruses tested. None of the sera tested contained antibodies to Araguari, EEE, yellow fever, Ilheus, Sp H34675, Marituba, Caraparu, Catú, Guaroa, or Tocaiuma viruses. Antibody to Icoaraci and Itaporanga viruses were found most frequently in the mammal blood (Tables 4 and 5).

The whole blood and organ specimens collected from the mammals are currently being processed for virus isolation in the virus laboratory at the Evandro Chagas Institute, and results are not yet available. The whole blood smears taken from 472 animals have been examined and sorted, although the suspected positive slides have not, as yet, been confirmed. Preliminary results indicate that no anthrax has been found, while microfilariae have been tentatively identified on 59 slides, and trypanosomes on 17 slides.

COMMENT: Results from the mammal collecting program are available for June-August 1977. Additional collections, presently being conducted, will provide sufficient information to decide on the need to change the emphasis in the collecting effort, e. g. more arboreal animals - monkeys, etc. The bird collecting program, initiated in September 1977, should provide much valuable information and could eventually be a program of special emphasis. Plans are being made to conduct viremia studies, following experimental infection with Oropouche virus, in selected birds and mammals. Main effort will be to conduct these studies on species that are most frequently found with antibodies to Oropouche virus in nature.

Table 1

Numbers of Mammals Collected by Trapping in the Curuá Una Study Site, Pará, Brazil, 15 June-31 August, 1977.

Species	Number Trapped per Month			
	June	July	August	Total
<b>Marsupialia</b>				
<u>Monodelphis brevicaudata</u>	-	2	-	2
<u>Marmosa cinerea</u>	2	1	-	3
<u>M. murina</u>	1	1	-	2
<u>M. parvidens</u>	-	1	-	1
<u>Metachirus nudicaudatus</u>	3	4	-	7
<u>Didelphis marsupialis</u>	5	3	1	9
<b>Rodentia</b>				
<u>Oryzomys bicolor</u>	4	7	-	11
<u>O. capito</u>	2	4	1	7
<u>O. concolor</u>	1	7	-	8
<u>Rhipidomys</u> sp.	-	1	-	1
<u>Proechimys guyannensis</u>	7	11	10	28
<u>P. longicaudatus</u>	15	27	12	54
<u>P. sp.</u>	-	1	-	1
<u>Mesomys hispidus</u>	-	2	1	3
<b>Total</b>	<b>40</b>	<b>72</b>	<b>25</b>	<b>137</b>
<b>Trap nights</b>	<b>1,301</b>	<b>3,080</b>	<b>2,860</b>	<b>7,241</b>
<b>Mammals/100 trap nights</b>	<b>3.07</b>	<b>2.34</b>	<b>.87</b>	<b>1.89</b>

/zcm.

Table 2

Numbers of Bats Collected With Mist Nets in the Curuá Una Study Site, Pará, Brazil, 15 June-31 August, 1977.

Species	Number Mist Netted per Month			
	June	July	August	Total
<u>Sacopteryx leptura</u>	-	-	1	1
<u>Peropteryx macrotis</u>	1	-	-	1
<u>Pteronotus parnellii</u>	7	3	2	12
<u>Tonatia</u> sp.	1	1	2	4
<u>Phyllostomus discolor</u>	-	-	1	1
<u>P. elongatus</u>	-	2	-	2
<u>P. hastatus</u>	-	2	-	2
<u>P. latifolius</u>	1	-	-	1
<u>Glossophaga</u> sp.	-	2	-	2
<u>Lionycteris spurrelli</u>	-	1	-	1
<u>Lonchophylla thomasi</u>	-	1	-	1
<u>Lonchophylla</u> sp.	-	1	-	1
<u>Carollia brevicauda</u>	22	65	90	177
<u>Sturnira tildae</u>	-	7	6	13
<u>S. lilium</u>	-	18	33	51
<u>Vampyrops hellers</u>	-	6	1	7
<u>Artibens cinereus</u>	-	5	3	8
<u>A.</u> sp.	-	8	4	12
<u>Desmodus rotundus</u>	1	2	1	4

Table 2. Continued

<u>Stenoderminae</u> sp. A	2	4	2	8
<u>Stenoderminae</u> sp. B	1	1	-	2
<u>Phyllostomidae</u> sp. A	-	-	1	1
<b>Totals</b>	<b>36</b>	<b>129</b>	<b>147</b>	<b>312</b>
<b>Net hours</b>	<b>69</b>	<b>170</b>	<b>278</b>	<b>517</b>
<b>Bats per net hour</b>	<b>.52</b>	<b>.76</b>	<b>.53</b>	<b>.60</b>

/zcm.

Table 3

Numbers of Mammals Collected by Hunting in the Curuá Una Study Area, Pará, Brazil, 15 June-31 August 1977.

Species	Number Collected by Hunting per Month			
	June	July	August	Total
Primates				
<u>Callicebus torquatus</u>	-	1	-	1
<u>Alouatta belzebul</u>	-	1	-	1
Edentata				
<u>Tamandua tetradactyla</u>	-	2	-	2
Rodentia				
<u>Agouti paca</u>	-	1	-	1
<u>Dactylomys</u> sp.	-	-	1	1
Carnivora				
<u>Nasua nasua</u>	-	-	2	2
<u>Felis wiedii</u>	-	-	1	1
Totals	0	5	4	9

/zcm.

Table 4  
Numbers of Positive Mammal Sera for Antibodies to 9 Arboviruses. Results From HI Tests  
Conducted on Mammal Sera Collected near Curuá Una, Pará, Brazil, 1977.

Species	WEF	Mayaro	Mucambo	St. Louis	Guama	Icoaraci	Itaporanga	Droopunche	Utinga	Total Tested
<u>Marsupialia</u> <u>Philander opossum</u>	-	-	-	-	1	-	-	-	-	21
<u>Primates</u> <u>Callicebus torquatus</u> <u>Alouatta belzebul</u>	1	1	1	-	-	-	-	1	1	1
<u>Edentata</u> <u>Tamandua tetradactyla</u>	-	-	-	1	-	-	-	-	-	2
<u>Rodentia</u> <u>Proechimys guyannensis</u> <u>P. longicaudatus</u>	-	-	-	-	1	15	7	2	-	24
<u>Totals</u>	1	2	1	1	2	22	12	1	1	100

/Zcm.

Table 5

Numbers of Positive Sera for Antibodies to WEE, Mayaro, Mucambo, St. Louis, Guama, Icoaraci, Itaporanga, Oropouche, and Utinga Viruses. Results from HI Tests Conducted on Mammal Sera Collected Near Curuá Una, Pará, Brazil, 1977.

Group	Positive	Negative
Marsupials	1	21
Bats	0	259
Monkeys	2	2
Edentates	1	2
Rodents	25	92
Carnivores	0	2
Totals	29	378

/zcm.

## B. FIELD SURVEILLANCE FOR SYLVATIC VECTORS OF OROPOUCHE VIRUS

OBJECTIVE: To conduct field collections of hematophagous insects in forested areas with specific aims to:

1. Incriminate the natural vector(s) of Oropouche virus in the sylvatic environment.
2. Determine the ecology of incriminated vector species.
3. Document host feeding preferences of insects found capable of transmitting Oropouche virus.

BACKGROUND: In May 1977 a field surveillance program for hematophagous insects was initiated in the forested region of Curuá Una, Pará, Brazil. This study site is located 44 km south and 40 km west of Santarém. Both Mojui dos Campos, located between Curuá Una and Santarém, and Santarém were involved in epidemics of Oropouche in 1975. Data obtained from field epidemiological investigations during the epidemics strongly indicated that Oropouche virus was insect borne (see Annual Report for 1976).

Due to the irregular and sporadic pattern in occurrence of epidemics of Oropouche virus in the Amazon basin, it seems probable that the disease is maintained in a sylvatic vertebrate-insect cycle of transmission. This is supported by serologic evidence of Oropouche activity among wild birds and mammals. Moreover, insect transmission of Oropouche virus from viremic animals to susceptible hosts has been documented during this reporting period.

DESCRIPTION: The field surveillance program was designed to monitor blood feeding insects for virus activity and to obtain basic entomological information concerning vector ecology. Entomological information is being gathered by using multiple collecting techniques (animal bait traps, light traps, shannon traps, suction traps and man biting collections) to capture medically important insects. Insect captures are being conducted in both the light and dark phases of the day; at ground and canopy levels. When sufficient data are available for selecting promising species as vectors of Oropouche virus, the species will be evaluated in the laboratory to delineate vector competence.

PROGRESS: Preliminary results from the surveillance program are listed in table 1. Since these studies were initiated during the

onset of the dry season (May-September), the populations of most species were low. The largest numbers of insects, collected to date, were from the Culicinae and Phlebotominae groups; however, all hematophagous insects are being processed for virus isolation. Tables 1 and 2 give a preliminary list of collection methods and species collected. A routine collecting schedule is also provided (Table 3).

Table 1

Numbers Collected by Insect Group, Habitat, Time of day, Month and Collection Method in Forest Sites at Curuá Una, Pará, Brazil, 1977.

Months Insect Groups	Man Biting Collections				Shannon Traps	Light Traps		
	Ground		Canopy					
	Day	Night	Day	Night				
May								
Anophelinae	20	3	-	-	5	2		
Culicinae	743	17	-	-	293	290		
Phlebotominae	294	161	-	-	260	276		
Ceratopogonidae	8	1	-	-	154	125		
June								
Anophelinae	14	2	-	2	7	6		
Culicinae	643	29	39	20	190	890		
Phlebotominae	249	189	-	13	760	911		
Ceratopogonidae	45	6	-	13	300	301		
July								
Anophelinae	15	3	-	1	6	8		
Culicinae	187	26	56	23	90	437		
Phlebotominae	183	164	-	13	169	745		
Ceratopogonidae	11	2	-	2	24	127		
August								
Anophelinae	7	21	-	1	23	27		
Culicinae	227	137	71	51	818	1707		
Phlebotominae	98	170	-	11	246	1225		
Ceratopogonidae	-	1	-	-	-	5		

/zcm.

Table 2

Preliminary List of Mosquito Species Collected by Multiple  
Collecting Methods in Forest Areas of Curuá Una, Pará, Brazil,  
1977.

<u>Aedeomyia squamipennis</u>	<u>Psorophora (Jan.) ferox</u>
<u>Aedes (How.) fulvithorax</u>	<u>Limatus durhamii</u>
<u>Aedes (Fin.) fluviatilis</u>	<u>Limatus flavisetosus</u>
<u>Aedes (Och.) fulvus</u>	<u>Limatus pseudomethysticus</u>
<u>Aedes (Och.) serratus</u>	<u>Sabethes (Sab.) cyaneus</u>
<u>Aedes (How.) septemstriatus</u>	<u>Sabethes (Sab.) glaucodaemon</u>
<u>Anopheles (Ano.) mattogrossensis</u>	<u>Trichoprosopon (Trc.) digitatum</u>
<u>Anopheles (Ano.) mediopunctatus</u>	<u>Wyeomyia (Den.) aporronoma</u>
<u>Anopheles (Nys.) nuneztovari</u>	
<u>Anopheles (Nys.) oswaldoi</u>	<u>Anopheles (Nys.) sp.</u>
<u>Anopheles (Nys.) triannulatus</u>	<u>Culex</u> spp.
<u>Culex</u> spB#21	<u>Culex (Carrollia)</u> sp.
<u>Culex (Cux.) corniger</u>	<u>Culex (Mel.)</u> sp.
<u>Culex (Cux.) coronator</u>	<u>Haemagogus</u> spp.
<u>Culex (Cux.) declarator</u>	<u>Psorophora</u> spp.
<u>Culex (Mel.) vomerifer</u>	<u>Uranotaenia</u> spp.
<u>Mansonia (Man.) titillans</u>	<u>Limatus</u> spp.
<u>Psorophora (Jan.) albipes</u>	<u>Wyeomyia</u> spp.

/zcm.

Table 3  
Field Collecting Schedule - Curuá Una

Type of Collection	Collection Time
1. Light traps	18:00 - 06:30
2. Man-biting - group level	09:00 - 09:30
3. Man-biting - Canopy level	09:45 - 10:15
4. Man-biting - group level	15:00 - 15:30
5. Man-biting - Canopy level	15:45 - 16:15
6. Man-biting - group level	17:30 - 18:30
7. Shannon trap	19:30 - 20:30
8. Man-biting - Canopy level	21:00 - 21:30
9. Man-biting - group level	21:35 - 22:05
 <b>*Rotation of Sentinel Animals - Hamsters</b>	
1. Diurnal animal group	06:30 - 17:30
2. Night animal group	18:00 - 06:00

\* Deployment of sentinel animals commenced in August, 1977 and no results are available at this time.

/zcm.

C. LABORATORY TRANSMISSION STUDIES - OROPOUCHE VIRUS

OBJECTIVE:

1. To ascertain the infectivity of Oropouche virus to Culicoides paraensis (Goeldi), Culex pipiens quinquefasciatus (Say) and other hematophagous insects.
2. To determine if these insects can transmit Oropouche virus biologically and/or mechanically from viremic hamsters to susceptible hamsters.
3. To determine the extrinsic incubation period of the virus in suspect vectors.

BACKGROUND: Data obtained from epidemiological investigations indicate that Oropouche virus probably has two distinct cycles; an endemic sylvatic cycle and an epidemic urban cycle. Serological surveys have provided evidence of Oropouche virus activity among forest dwelling vertebrates; however, no endemic vector has been identified. Investigations of urban outbreaks suggest that blood-feeding insects play a significant role in virus transmission during epidemics. Therefore, laboratory transmission studies were initiated to identify potential urban and sylvatic vectors of Oropouche virus.

DESCRIPTION: General. Species representing 4 families of hematophagous insects (Culicidae, Ceratopogonidae, Triatomidae and Psychodidae) have been tested for their ability to transmit Oropouche virus. All species of insects, except for Culicoides paraensis (Ceratopogonidae), were reared under laboratory conditions ( $26.5^{\circ}\text{C} \pm 1.95\% \text{ RH}$  and 12/12 light/dark photo period). A laboratory colony of Cx. p. quinquefasciatus (Culicidae) was established in 1975 from mosquito collections conducted in Belém, Pará, Brazil and this colony supplied the mosquitoes used in the transmission studies. Since a colony has not been established for C. paraensis, all adult Culicoides used in the studies were collected from an agriculture experiment station near Belém.

Populations, for testing, of Triatomidae and Psychodidae were obtained from the colonies established at the Institute of Evandro Chagas, Belém, Brazil by members of the Wellcome Parasitology Unit.

Mosquitoes and midges were maintained on a 10% sugar solution; however, the solution was removed from the cages 6 hr prior to exposing experimental animals to the test populations.

Oropouche virus, strain Br An 19991, was used to infect the

experimental animals.

Young hamsters (23-25 days of age) were used as the donor and recipient vertebrate hosts in all transmission experiments. Hamsters infected with Oropouche virus generally produce a high virus titer followed by death in 72 to 96 hours. However, seroconversion to Oropouche virus has been documented among surviving hamsters.

**Virus Titration.** Hamsters (23-25 days old) were inoculated intracerebrally (IC) with 0.1 ml of undiluted hamster serum containing Oropouche (Br An 19991) virus. Twenty to 24 hrs following the IC inoculation, 0.1 ml of blood was obtained from the hamster by cardiac puncture and immediately added to 0.9 ml of PBS containing 0.4% bovine albumin. This 1:10 dilution was further diluted and titrated in Vero cells to determine the titer of Oropouche virus in the donor hamster. After bleeding, the hamsters were immediately exposed to populations of insects included in the transmission tests. In some experiments, the viremia level was retested 3-4 hrs after the hamster was exposed to the insects.

**Virus Isolation and Identification.** Individual insects were homogenized and suspended in phosphate-buffered physiological saline (PBS) containing bovine albumin and antibiotics. A 1.5 ml aliquot of the diluent was used for Culex mosquitoes and sandflies and 1.0 ml was used for Culicoides.

After centrifugation of the insect homogenate at 1500 RPM, the supernatant fluid was aspirated and 0.1 ml (of the supernatant) was inoculated into each of 3 tubes of Vero cells. The tubes were observed microscopically every 2 days to detect viral cytopathic effect (CPE). Tubes demonstrating a 3 to 4 + CPE were harvested and frozen at -60°C. To identify the virus, a 1:100 dilution of the infected fluid was mixed with equal amounts of Oropouche hyperimmune mouse ascitic fluid, incubated for 1 hour at 37°C and assayed for infectivity. These tests were performed in microtiter plates to which Vero cells were added after incubation of the virus and virus-serum mixtures. The test control series consisted of infected fluids without additions of the Oropouche virus hyperimmune mouse ascitic fluids. The tests were routinely read 3-4 days post-inoculation or when the virus control showed a 3-4 + CPE.

PROGRESS:

Transmission Tests With Culex quinquefasciatus

Mechanical Transmission. Mosquitoes were fed on a viremic

hamster ( $10^{7.0}$ SMLD<sub>50</sub>/0.02 ml of blood) during the dark phase of the photo period. Feeding mosquitoes were removed prior to full engorgement and transferred to a 2nd holding cage. Following the transfer, a susceptible hamster was exposed and the mosquitoes were allowed to feed to repletion. The hamster was removed and observed for 3 weeks for signs of illness. No evidence of Oropouche virus infection was detected in the recipient hamster.

**Biological Transmission.** Three separate attempts were made to demonstrate biological transmission of Oropouche virus by Cx. quinquefasciatus. In the initial attempt, mosquitoes were fed on a hamster circulating  $10^{7.0}$ SMLD<sub>50</sub>/0.02 ml of Oropouche virus. Engorged mosquitoes were removed and held until tested for virus transmission. Susceptible hamsters were exposed to the Cx. quinquefasciatus population on days 0, 1, 5, 10 and 17 post-infectious blood meal. Several mosquitoes were removed on each of these days and assayed, individually, for virus. Oropouche virus was recovered from 1 of 5 mosquitoes individually assayed on day 1 (Table 1). Virus was not recovered from any of the other mosquitoes tested; nor was virus successfully transmitted to susceptible hamsters.

In the 2nd experiment, 2 groups of mosquitoes were fed on viremic hamsters. The 1st group was fed on a hamster circulating  $10^{8.2}$ SMLD<sub>50</sub>/0.02 ml of Oropouche virus, and the 2nd group on a hamster circulating  $10^{8.0}$ SMLD<sub>50</sub>/0.02 ml. Mosquitoes were then allowed to feed on susceptible hamsters at various periods post infection. Insects from this study were not assayed for virus isolation.

Results of this experiment are presented in Table 2. A considerable number of mosquitoes fed on each of the susceptible hamsters exposed on days 2, 4, 6, 8, 10, 12, 14 and 16. One hamster, exposed on day 8, seroconverted to Oropouche virus.

In the final experiment (Test No. 3) with Cx. quinquefasciatus, 3 day old mosquitoes were fed upon a viremic hamster circulating  $10^{8.0}$ SMLD<sub>50</sub>/0.02 ml of Oropouche virus and 4 day old mosquitoes were fed on a hamster circulating  $10^{7.8}$ SMLD<sub>50</sub>/0.02 ml of virus. Mosquitoes exposed to viremic hamsters were allowed to engorge on non-infected hamsters on days 15, 16 and 21.

Results of this experiment are presented in table 3.

Oropouche virus was recovered from 1 hamster exposed to infected mosquitoes on day 21. Thirty mosquitoes were assayed individually from this group and only a single, non-engorged individual was found positive for Oropouche virus. On day 22, 15

Table 1

Test No. 1. Results of Transmission Test With Culex quinquefasciatus (Say) fed on a Hamster Circulating  $10^{7.0}$  SMLD<sub>50</sub>/0.02 ml of Oropouche Virus.

Days After Infectious Blood Meal	Virus Isolation/ Number of Mosquitoes Tested	Transmission Results in Hamsters
0	0/5	Neg.
1	1/0	Neg.
5	0/5	Neg.
10	0/5	Neg.
17	0/8	Neg.

/zcm.

Table 2  
Test No. 2. Transmission of Oropouche Virus to Hamsters by Culex quinquefasciatus (Say).

Days After Infectious Blood Meal	Virus Titer of Infectious Blood Meal				Transmission Results	Number of Mosquitoes Fed	Number of Mosquitoes Fed	Transmission Results
	10 <sup>8.2</sup> SMLD <sub>50</sub> /0.02 ml		10 <sup>8.0</sup> SMLD <sub>50</sub> /0.02 ml					
	Number of Mosquitoes Fed	Transmission Results	Number of Mosquitoes Fed	Transmission Results				
2	14	-	-	-		17	-	-
4	24	-	-	-		30	-	-
6	28	-	-	-		33	-	-
8	21	+ <sup>a</sup>	-	-		19	-	-
10	4	-	-	-		8	-	-
12	5	-	-	-		14	-	-
14	17	-	-	-		23	-	-
16	9	-	-	-		13	-	-

<sup>a</sup> Oropouche infection in test hamster confirmed by serological test.  
/zcm.

Table 3  
Test No. 3. Transmission of Oropouche Virus to Hamsters by Culex quinquefasciatus\*.

Days After Infectious Blood Meal	Virus Titer of Infectious Blood Meal			Transmission Results	Number of Bites	Number of Hamsters Infected	Transmission Results
	10 <sup>8</sup> .0 SMLD <sub>50</sub> /0.02 ml	10 <sup>7</sup> .8 SMLD <sub>50</sub> /0.02 ml	10 <sup>7</sup> .6 SMLD <sub>50</sub> /0.02 ml				
15	49	-	-		50	-	
16							
21	10	-	-		33	+ <sup>a</sup>	

\* Mosquitoes were 3-4 days old when exposed to viremic hamsters  
<sup>a</sup> Oropouche virus infection confirmed by virus isolation.

/2cm.

mosquitoes (10 engorged, 5 non-engorged) were assayed individually for Oropouche virus. None of the mosquitoes were positive for virus. In addition, none of the hamsters exposed to this subgroup of mosquitoes exhibited signs of virus infections.

#### Transmission Tests With *Culicoides paraensis*

Mechanical Transmission. Techniques used to demonstrate mechanical transmission with *C. paraensis* were similar to those used with *Cx. quinquefasciatus*. Since *C. paraensis* are day-biters, all experimental feedings were conducted during the day photo-period. The resulting data indicated that *C. paraensis* do not transmit Oropouche virus mechanically under the test conditions. This was substantiated by interrupted blood feeding of midges on infected hamsters with a high viremia ( $10^7.0$  SMLD<sub>50</sub>/0.02 ml) and the refeeding of 19 and 28 midges on susceptible hamsters at 1½ hrs and 17 hrs, respectively. Oropouche virus was not detected in test hamsters during the 3 week laboratory observation period.

Biological Transmission. The 1st preliminary test for biological transmission of Oropouche virus by *C. paraensis* resulted in the death of 4 hamsters which had been exposed to *C. paraensis* that had fed on a viremic hamster (with a viremia of  $10^7.0$  SMLD<sub>50</sub>/0.02 ml of blood). Unfortunately, samples were not taken from these animals since they died before blood could be obtained. Therefore, it could only be assumed that Oropouche virus was the cause of death of the test animals. However, sub-samples of the *Culicoides* used for this test, taken at various intervals after the infectious blood meal, demonstrated that 46% (17/37) of the midges were positive for Oropouche virus (Table 4).

A second study was initiated to demonstrate biological transmission of the virus (Table 5). The results of this study documented biological transmission with isolation of Oropouche virus from 2 hamsters which had been exposed to *C. paraensis* after 8 and 9 days extrinsic incubation. In addition to the positive transmission of Oropouche virus by *C. paraensis*, it is interesting that only 1 blood feeding, on day 9, from an infected midge resulted in successful transmission of Oropouche virus (Table 5).

Attempts were made, during this test, to isolate virus from individual specimens of *C. paraensis*. Fifty per cent of the specimens removed immediately after engorgement on the hamster, containing  $10^6.0$  SMLD<sub>50</sub>/0.02 ml of virus, were positive for Oropouche virus. All subsequent attempts at virus isolation were

Table 4

Test No. 1. Summary of Results of Attempts to Transmit Oropouche Virus to Susceptible Hamsters by Culicoides paraensis fed on Infected Hamsters Circulating 10.0 SMLD<sub>50</sub>/0.02 ml of Oropouche Virus, and Virus Isolation From Samples of Midges Taken at Various Intervals Following Viremic Blood Meal.

Days After Infectious Blood Meal	Number Fed/Hamster	Transmission Results	Virus Isolation/ Midges Tested
0	21	-	3/3
1	10	-	0/7
2	7	-	2/6
3	7	-	3/3
4	4	**	5/7
5	1	**	3/6
6	3	**	1/5
7	1	**	
8	1	**	
9	1	**	

\*Hamsters died within 5-7 days following feeding, which is characteristic of Oropouche virus infection in hamsters; however, Oropouche virus was not confirmed as the cause of death.  
/zcm.

Table 5

Test No. 2. Summary of Results of Attempts to Transmit Oropouche Virus to Susceptible Hamsters by Culicoides paraensis fed on Infected Hamsters Circulating 10<sup>6</sup>.0 SMLD<sub>50</sub>/0.02 ml of Oropouche Virus and Virus Isolations from Samples of Midges Taken at Various Intervals Following Viremic Blood Meal.

Days After Infectious Blood Meal	Number Fed/Hamster	Transmission Results	Virus Isolation/ Midge Tested
0	28	-	5/10
1	31	-	-
2	24	-	0/ 9
3	38	-	0/ 3
4	3	-	0/ 5
5	13	-	0/15
6	3	-	-
7	11	-	0/ 3
8	5	+	0/ 7
9	1	+	-
10	0	-	0/ 8
11	0	-	0/16

72

72cm.

negative, although virus was transmitted to hamsters by the test population (Table 5).

The 3rd and 4th experiments (Tables 6 and 7) reconfirmed biological transmission of Oropouche virus by C. paraensis. It is worthy to note that virus was transmitted to the hamsters by a single bite each and, therefore, repeats the observations made in the 2nd test. Data obtained in the 3rd study indicates that virus was transmitted to hamsters by Culicoides midges 8 and 9 days after infective blood meals, while Oropouche was transmitted to hamster as early as day 4 and 5 in the 4th test. The most important variable between the two experiments (# 3 and # 4) appears to be the viremia of the infective blood meal; however, additional studies are needed.

When comparing the isolation of Oropouche virus in the 2 experiments (numbers 3 and 4) it was noted that virus was not recovered from any of the Culicoides midges in the 3rd experiment, while 20.5% (26/127) of the C. paraensis tested in the 4th experiment were positive for Oropouche virus.

#### Oropouche Transmission Tests With Triatomids (Kissing Bugs) and Sandflies

Preliminary transmission studies are being conducted with 4 species of Triatomidae (Triatoma infestans, Triatoma brasiliensis, Rodnius prolixus, Triatoma sordida) and 2 species of Psychodidae (Lutzomyia longipalpis and Lutzomyia flaviscutellata) to determine if these species are capable of becoming infected and transmitting Oropouche virus.

The 4 species of Triatomidae were allowed to feed on viremic hamsters having virus titers ranging from  $10^{6.0}-7.5 \text{ SMLD}_{50}/0.02 \text{ ml}$  of blood. Each species was allowed to refeed on susceptible hamsters on days 11, 32 and 52 post infective blood meal. After 3 weeks, none of the experimental hamsters demonstrated signs of Oropouche infection.

Transmission studies being conducted with the 2 species of Psychodidae are incomplete; however, the initial results do not indicate Oropouche virus transmission.

COMMENT: Laboratory studies conducted with Cx. quinquefasciatus and C. paraensis have failed to demonstrate mechanical transmission of Oropouche virus. However, biological transmission of Oropouche virus by Cx. quinquefasciatus and C. paraensis has been documented by reisolation of the virus in susceptible hamsters exposed to feeding populations of these

Table 6  
 Test No. 3. Summary of Results of Attempts to Transmit Oropouche Virus to Susceptible Hamsters  
 by Culicoides paraensis fed on Infected Hamsters Circulating 10<sup>6</sup>-5SMLD<sub>50</sub>/0.02 ml of Oropouche  
 Virus and Virus Isolations From Samples of Midges Taken at Various Intervals Following Viremic  
 Blood Meal.

Days After Infectious Blood Meal (or hours)	Number Fed/Hamster	Transmission Results		Virus Isolation/ Midges Tested
		1-1½ hrs.	19*	
6 hrs.	17*	-	-	-
1	17	-	-	0/5
2	14	-	-	0/1
3	5	-	-	0/2
4	1	-	-	-
5	3	-	-	0/1
6	0	-	-	-
7	2	-	-	0/1
8	1	+	-	0/2
9	1	+	-	0/5
10	0	-	-	0/8

\* Interrupted feedings to demonstrate mechanical transmission.  
 /zcm.

Table 7

Test No. 4. Laboratory Transmission Studies of Oropouche Virus by Culicoides paraensis Using Hamsters as Test Animals.

Days After Infectious Blood Meal	Virus Titer of Infectious Blood Meal			Number Fed	Transmission Results	$10^7.2 \text{ SMLD}_{50}/0.02 \text{ ml}$	Transmission Results
	$10^8.2 \text{ SMLD}_{50}/0.02 \text{ ml}$		Number Fed				
1	50	-	-	65	-	-	-
2	69	-	-	90	-	-	-
3	30	-	-	36	-	-	-
4	22	-	-	27	+ <sup>a</sup>	+ <sup>a</sup>	-
5	7	-	-	9	+ <sup>a</sup>	-	-
6	8	-	-	11	-	-	-
7	3	-	-	4	-	-	-
8	1	-	-	2	+ <sup>a</sup>	+ <sup>a</sup>	-

<sup>a</sup> Determined by isolation of Oropouche virus.  
/zcm.

Table 8

Test No. 4. Results of Oropouche Virus Isolation Test With Culicoides paraensis After Feeding on an Infected Hamster.

Days After Infectious Blood Meal	Virus Titer of Infectious Blood Meal 10 <sup>8.2</sup> SMLD <sub>50</sub> /0.02 ml	Virus Titer of Infectious Blood Meal 10 <sup>7.2</sup> SMLD <sub>50</sub> /0.02 ml
1	4/5* = 80%	7/8 = 87%
2	0/7	1/7 = 14%
3	0/8	3/8 = 37%
4	1/8 = 12%	0/8
5	0/8	1/8 = 12%
6	0/5	1/7 = 14%
7	1/5 = 20%	0/5
8	2/8 = 25%	2/6 = 33%
9	0/8	3/8 = 37%

\* Number of Oropouche virus isolation/Number of Culicoides tested.  
/zcm.

insects in the laboratory. Available data indicates that C. paraensis is a more efficient vector than Cx. quinquefasciatus.

Biological transmission by Cx. quinquefasciatus was demonstrated to occur on 2 occasions. Eventhough, the successful transmission of Oropouche virus does demonstrate a vector potential, the low transmission and mosquito infection rates suggest that Cx. quinquefasciatus are not efficient vectors and probably do not contribute significantly to urban epidemics of Oropouche virus.

Notable inconsistencies were found in the results from attempts to isolate Oropouche virus from supposedly infected C. paraensis. It is germane to this subject to consider some of the factors having a direct influence on transmission test results with Culicoides.

First of all, the techniques and specialized equipment for working with infected midges are either not available or lack refinement. It has only been within recent years that research has documented the public health importance of Culicoides midges. Moreover, they are difficult to manipulate using established laboratory techniques because of their small size (< 1.0 mm).

The method employed to remove blood fed Culicoides from a viremic hamster was to aspirate the feeding population of C. paraensis as they engorged. Early removal of some of the feeding Culicoides was necessary to separate the small feeding population of midges from the larger non-feeding population. In addition, with several midges feeding in a relatively small area (the shaved stomach of a young hamster) and with variable stages of engorgement, it is difficult to obtain sufficient numbers of uniformly fed Culicoides for transmission studies. Therefore, some inconsistencies in test results are due to inadequate control techniques for obtaining fully engorged specimens.

Laboratory techniques are being reevaluated and modified to correct some of the inadequacies of the preliminary testing procedures. With more control of test paramenters, it is hoped that the information obtained from transmission tests will be improved.

Some inferences can be made by synthesis of the Oropouche transmission data and observations made on the biology of C. paraensis (see section D). Observations on mortality of field collected Culicoides indicate that more than 50% of the midges survive at least 8-9 days in the insectary. It should be noted that the test population was composed of members representing unknown ages. Information obtained from colonized material

indicates that female midges will accept a blood meal about 24 hrs after emergence. Oviposition data indicates that the average time for completion of the gonotrophic cycle in the laboratory is approximately 2-3 days. Oropouche transmission studies with hamsters demonstrated that Culicoides can transmit Oropouche virus 4-6 days after their initial infective blood meal. Therefore, if the 2nd blood meal occurs within 24-28 hrs after oviposition then one can assimilate enough information to establish a reasonable Oropouche virus transmission pattern (Table 9). The proposed transmission pattern does not account for the possibility of transovarian transmission of Oropouche virus.

It is apparent from available information that C. paraensis have sufficient longevity for Oropouche virus transmission. However, additional studies should be conducted to determine the longevity of C. paraensis under field conditions and to establish the frequency of blood feedings.

Table 9  
Proposed Transmission Pattern for Oropouche Virus by C. paraensis based on Laboratory Longevity Studies

Adult Longevity -Days																
0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Adult Emergence																
Infective Blood Meal																
Oviposition																
2nd Blood Meal																
Oviposition																
3rd Blood Meal																
<u>Extrinsic Incubation</u>																
<u>Oropouche Transmission Phase</u>																
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#### D. SPECIAL FIELD STUDIES WITH CULICOIDES PARAENSIS

OBJECTIVE: To describe the breeding sites of Culicoides paraensis in the field, parameters of host-seeking and blood engorgement and the seasonal activity in urban environs.

BACKGROUND: Previous investigations during epidemics of Oropouche virus indicate that C. paraensis are probably the vectors of this virus in urban areas. Therefore, special studies were designed to determine 1) the biting characteristics of this midge under field conditions, 2) to find/describe the preferred breeding habitats of C. paraensis, 3) to determine the season of peak abundance and 4) to quantify average blood meal size and time required for engorgement.

DESCRIPTION: In 1976 a surveillance program was initiated to find those areas producing high populations of C. paraensis in order to obtain sufficient numbers of these midges to conduct Oropouche transmission and preliminary colonization studies. A location meeting most of the experimental requirements was found at an agriculture experimental research station (CEPLAC) located on the periphery of Belém, Pará, Brazil. This area was being utilized to investigate cocoa and banana tree production and had been established for a number of years. Man-biting collections conducted at this site revealed that a dense population of C. paraensis was active in a relatively small area, near a group of buildings.

Due to the large concentration of the biting midge population near the buildings, it was speculated that the breeding sites would also be found close to the area. Therefore, a transect study was conducted to establish the spatial distribution of biting activity and surveillance for breeding sites was initiated to find the preferred breeding habitats.

Transect Studies. In order to define the distribution of biting activity in the experimental area, a transect study was conducted. Preliminary collection data indicated that midge biting activity near one of the houses was intense; therefore, this site was chosen as the focus for the transects (Figure 1). The house nearest the center of the transects is utilized as a staging site for employees involved in maintaining the experimental cocoa and banana trees. Therefore, variable human activity in or near the house occurs only during the day. A team of 2 collectors with mouth aspirators were positioned at 4 locations separated by 20 m along the transect. Biting midges were aspirated from the collectors exposed legs for a collecting period of 15 minutes. The Culicoides collected by each team were enumerated and the 2-man teams would then systematically rotate to another collecting

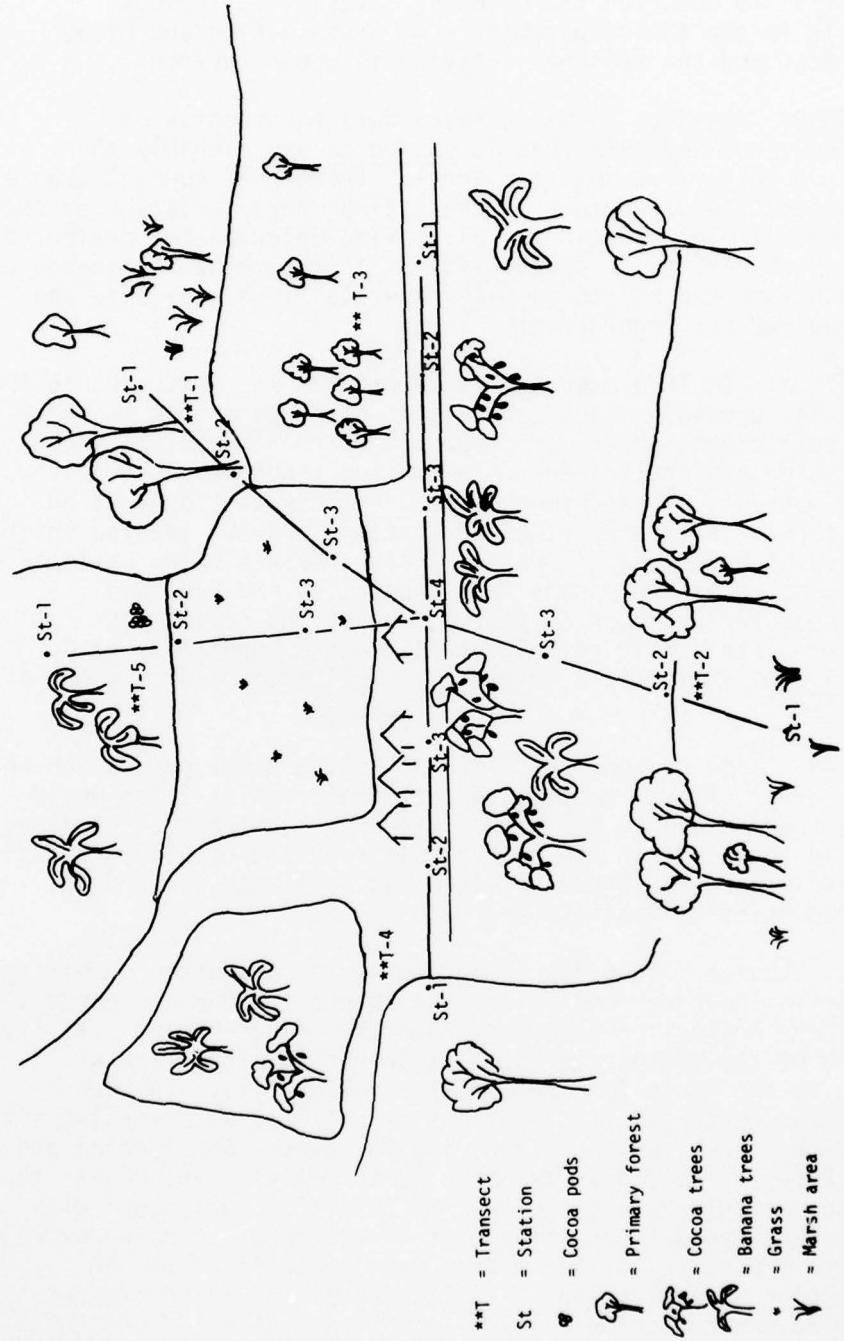


Fig. 1 Illustrates the design of the transect study and the relationship of the habitat types.

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point along the same transect to repeat the collection routine. A series of 5 transects were conducted.

Breeding Site Surveillance. Emergent trap studies are being conducted to determine the preferred breeding sites for C. paraensis. This program is being accomplished by monitoring a diversity of potential breeding habitats by various types of emergent traps. A brief description of the habitats monitored and types of emergence traps employed are illustrated by figures 2-10.

Seasonal Abundance. Collections for C. paraensis are being conducted near houses in 2 districts of Belem, Brazil. Captures are conducted for 4 consecutive 30 min. intervals, from 1400-1600 hrs., at each house for 2 consecutive days on alternate weeks. Two collectors are employed for each capture and they aspirate the biting midges only from their exposed legs. This study was initiated in July, 1977 and will be continued for a full 12 month period.

Size of Blood Meal and Engorgement Time. The study was conducted by allowing individuals of field populations of C. paraensis to feed at liberty on the exposed legs of 4 collectors. When a midge was observed to alight and begin to feed, a small glass tube was placed over the feeding midge. Following the voluntary withdrawal from the host, the glass vial was stoppered. The engorgement time was recorded for each specimen. Engorged midges were killed by chloroform fumes and grouped into units of 10 for weight measurements. Similar capture techniques were used with midges that were not permitted to probe and feed. All specimens were weighed with a Cahn model 4400 Electro balance R.

PROGRESS: Transect Studies. Results from studies to determine the spatial distribution of host-seeking C. paraensis revealed a concentration of females within a 20 m radius of the transect focus, near the house (Figure 11). The number of C. paraensis recorded at collecting station No. 4 was considerably higher than those recorded for station No. 3, which was located 20 m away. Data recorded for transects 1, 2, 3, and 4 exhibited low biting activity for station Nos. 2 and 1. In addition, transect 5 was the only transect conducted with an increase in the biting activity for station No. 1, when compared to station Nos. 2 and 3. After completion of this study, it was discovered by emergence trapping that station No. 1 of transect 5 was located near a breeding habitat for several species of midges, including C. paraensis. Therefore, the composite data indicates that the close proximity of the breeding site, decomposing cocoa pods, could account for the higher number of C. paraensis recorded for this station. This does not appear to be the case for station No. 4.

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Figure 2. Macro habitat of breeding sites for Culicoides paraensis (Goeldi).

Figure 3. A banana stump begins to decay and serves as a breeding site for Culicoides paraensis.

Figure 4. Discarded banana stalks may be the largest producers of emerging adult Culicoides paraensis in the peridomiciliary environment.

Figure 5. Emergence trap on a banana stump.

Figure 6. Cocoa pods are natural and receptacles that are sometimes used by female Culicoides paraensis for oviposition sites.

Figure 7. A collection of cocoa pods in the field.

Figure 8. An emergence trap over cocoa pods.

Figure 9. An emergence trap for sampling swamp areas.

Figure 10. An emergence trap for sampling leaf litter and soil habitats.



Figure 2



Figure 3

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Figure 4



Figure 5

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Figure 6



Figure 7



Figure 8



Figure 9

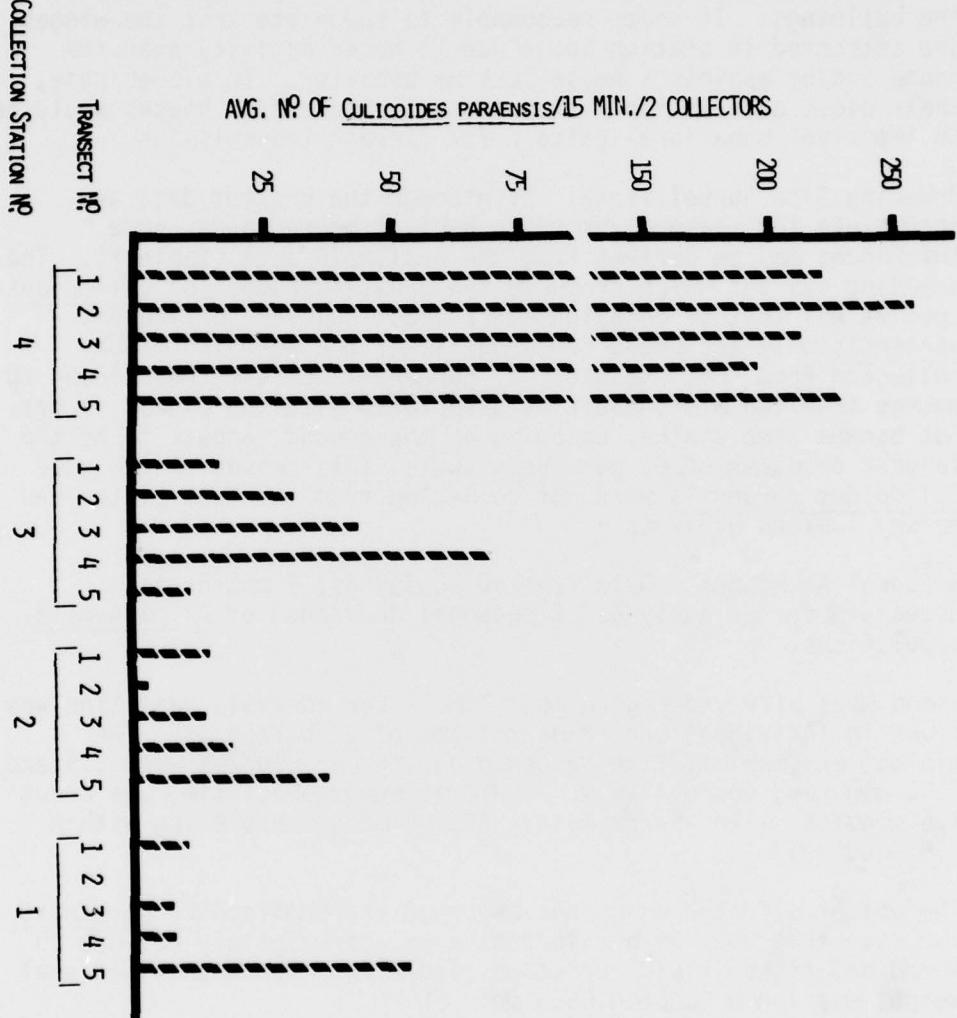
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Figure 10

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Figure 11 . Monitoring of C. paraensis biting activity and distribution by means of a transect study. Data represents 5 transects with 4 collecting stations (20 m apart)



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Emergence data indicates that there exist some breeding habitats (banana stumps and stalks) within 20 m of station No. 4; however, the number of C. paraensis emerging from these sources would be inadequate to account for the concentrated number of midges near the buildings. It seems reasonable to speculate that the midges are attracted to station No. 4 due to human activity near the house and/or exhibit a house-seeking behavior. In either case, their close association with human activity and/or houses would be an important behavioral pattern for disease transmission.

Breeding Site Surveillance. Eventhough the present data are incomplete for seasonal breeding habitat preferences, some inferences may be derived from the available data (Table 1). The breeding habitat which produced the greatest number of Culicoides species was that of decaying cocoa pods; however, Culicoides paraensis only accounted for about 1% of the emerging midges collected from this habitat. C. paraensis has also been found to emerge from two micro-habitats associated with the banana trees. Cut banana tree stalks, decaying on the ground, appear to be the largest producer of C. paraensis during this season of the year. Culicoides paraensis were not collected from the leaf litter and marshy lowland habitats.

Seasonal Abundance. Data from an additional 9 months is necessary for an analysis of seasonal abundance of C. paraensis populations.

Blood Meal Size and Engorgement Time. Considerable variation was found in individual engorgement times of C. paraensis. The minimal and maximal time recorded during our studies were 0.5 and 5.00 minutes, respectively. The mean engorgement time was about 1.6 minutes, with approximately 90% of midges repleting within 2.5 minutes.

The weight differences of non-engorged and engorged C. paraensis indicate that body weight increases by approximately 88% due to blood and tissue fluid ingestion (Table 2). The mean blood meal weight was found to be 0.0523 mg.

Table 1  
Numbers of *Culicoides paraensis* (Goeldi) Collected in Emergence Traps by Habitat. Collections  
Conducted at CEPLAC, Belém, Brazil, 1977.

Month	Type of Habitat	Number of Trap Days	Total Number of <i>Culicoides</i> midges	Number of <i>C. paraensis</i>	% <i>C. paraensis</i> of Total Number Collected
June	A	884	13	2	15 %
	B	1222	4	-	-
	C	52	6,939	105	1.5%
	D	52	-	-	-
July	A	986	35	4	11 %
	B	1363	-	-	-
	C	58	5,951	66	1 %
	D	48	-	-	-
August	A	310	13	3	23 %
	B	620	-	-	-
	C	82	4,522	25	.5%
	D	62	54	54	100 %
	E	48	9	-	-
September	A	200	-	-	-
	B	400	-	-	-
	C	48	1,458	2	.1%
	D	49	641	641	100 %
	E	120	-	-	-

Habitats: A = Banana stumps; B = Ground leaf litter - banana and cocoa; C = cocoa pods-ground  
D = Cut banana tree stalks - ground; E = marshy lowland area - abundant leaf litter.  
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Table 2  
 Observations on the Blood Meal Size of Individual *Culicoides paraensis* Feeding on a Human Host,  
 Belém, Pará, Brazil, 1976.

No. Replicates	Average Weight (mg)	Minimum - Maximum Weights(mg)	Average Body Weight	Average Weight of Blood Meal	% Increase in Body Weight/Blood Meal
8*	0.5925	0.5255-0.7160	0.592	UNENGORGED	
8*	1.1147	0.0958-1.2590	0.1115	ENGORGED	88%

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 \* Each replicate included 10 *Culicoides*.  
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### E. OBSERVATIONS ON THE BIOLOGY OF CULICOIDES PARAENSIS

OBJECTIVE: To colonize Culicoides paraensis and document basic information on the biology of these biting midges.

DESCRIPTION: Biting midges (C. paraensis) used to initiate colonization studies were obtained from a experimental agriculture experiment station (CEPLAC, Executive Commission Plan of Agriculture and Cocoa), Belém, Pará, Brazil. Due to the availability of various breeding sites in this area, Culicoides paraensis could be collected through the year; however, highest population densities seemed to be found during the rainy season (November-May). Specimens for laboratory studies were collected by man-biting captures. Retaining containers for Culicoides were fabricated from 1/2 gallon ice cream paper cartons (Sealright<sup>R</sup>) modified with fine mesh screen tops to reduce midge mortality. When returned to the laboratory, the adult females were transferred to insectary maintenance containers. These containers were also constructed from 1/2 gallon cartons modified to facilitate reception of blood donor animals and oviposition containers.

Young hamsters (21-23 day old) were immobilized on a restraining board and were employed as the principal host for Oropouche virus transmission studies and for a blood source in the colonization efforts. Hamsters were prepared for midge feeding by shaving the stomach area. Oviposition containers were constructed from 50 ml wide mouth beakers and filter paper. The filter paper was saturated with water from decaying cocoa pods.

Larvae were reared in a transparent plastic container (L-26 cm, W-22 cm, D-11 cm). Rearing chambers were fitted with removable lids perforated with a center hole (7 cm) for placement of adult emergence collecting chambers and small lateral holes for aeration tubes. Aeration of the larval rearing media was necessary to reduce water surface film which interferes with adult emergence. Emergence chambers for adult midges were fabricated from pint plastic containers with inverted funnel inserts and fine mesh screen tops. Small cotton pads saturated with a 10% sugar solution were placed on the fine mesh screen tops to provide a carbohydrate source for newly emerging adults. Since C. paraensis adults are positive phototrophic, the rearing chambers were covered with black opaque plastic to exclude the light from the transparent sides of the containers. Thus, the only light in the rearing container was through the emergence chamber into which the adults would enter and be trapped. Adults are removed from the emergence containers on a routine schedule and maintained in holding cages at 26.5°C and 95% + RH. When necessary, CO<sub>2</sub> gas is

used to anesthetize adults for counting, transfers, etc.

Presently, the principal larval media is being formulated from processed rice hulls and routinely supplemented with substrate from cocoa pods and colonized nematodes (Anguillula silusiae). These nematodes are easily cultured in the laboratory on yeast and wetted oat-meal. Rice hulls are added to the rearing containers to a depth of 1 cm, then wetted with distilled water to a depth of 3-4 cm. The larval medium is prepared 2-3 days before midge eggs and/or larvae are introduced into the rearing media. Early formulation of the media allows for the natural multiplication of bacteria, protozoa, nematodes and algae which are food substances of the Culicoides larvae.

A preliminary study was conducted to determine the size of blood meal of C. paraensis maintained in the insectary. Hamsters were exposed to a feeding population of Culicoides. As the midges were leaving the host, engorged flies were collected and weighed. Prior to blood feeding, a sample of Culicoides were collected to determine the normal body weight. Each group of Culicoides was weighed on a Cahn Electrobalance R model 4400.

PROGRESS: Laboratory Blood Sources for Culicoides paraensis. The principle blood donor animals used to feed field and laboratory colonized midges have been young hamsters (21-23 days old). These animals are easy to maintain and the midges seem to fed relatively well on the shaved areas of hamsters. Blood meal ingestion studies have shown that Culicoides midges will imbibe similar quantities of blood when allowed to engorge on man or hamsters. Other animals used in preliminary blood donor attempts were rabbits, young chickens and white laboratory mice.

Midges were observed to feed on all of these animals; however, differences in host preferences were observed. Feeding trials with laboratory reared midges show a definite preference for human host as opposed to young hamsters.

Oviposition of C. paraensis. Blood fed C. paraensis begin laying eggs 2-3 days after blood ingestion. Eggs are oviposited individually in a single file as the female traverses a wet substrate. Present studies indicate that gravid females do not normally deposit eggs on the open water surface of containers. Quantitative data on egg production indicate that 48 to 86 eggs are deposited per adult female per gonotrophic cycle.

Egg Viability and Embryogenesis. Within 24 hrs. after eggs were oviposited in the oviposition containers, a random sample of the eggs were removed and evaluated for egg hatchability. The eggs were observed daily under a stereomicroscope. The greatest number

of eggs hatched 3-4 days post-oviposition (Figure 1). Approximately 85% of the eggs were viable.

Larval Rearing Media. Formulations of media evaluated for larval development and adult emergence were as follows:

1. Wood chips - In order to simulate rotting tree hole habitats, small wood fragments were obtained from a local saw mill and placed in a small plastic container to a depth of 1 cm. The substrate was then submerged in distilled water (2 cm deep) and allowed to decompose for several weeks to stabilize the organic composition and the biological fauna. Midge eggs and young larvae were then introduced into the medium.

2. Hay infusion - Hay of a unknown variety was loosely placed in the plastic containers with enough distilled water to cover the hay substrate. The hay infusion was prepared a few days before eggs and larvae were placed into the medium.

3. Leaf-litter infusion - Samples of decaying forest leaf-litter and plant material were formulated into a leaf-litter broth. The infusion was allowed to stabilize for a few days before use.

4. Cocoa pods - Decomposing cocoa pods were acquired from a Cocoa experimental research station. A few pods (1-2) were placed in the experimental rearing chambers in a horizontal position so that the added water would partially submerge the lower portion of the pods. Colonization material was placed in the chambers 2-3 days later.

5. Banana stalk infusion - Samples of deteriorating banana stalk were obtained and small quantities of the decaying material was added to distilled water and allowed to set for 3-4 days before the introduction of eggs and larvae.

6. Rice hull infusion - Processed rice hulls of a local variety were added to the rearing containers to a depth of 1 cm and soaked in 2-3 cm of water. This media was prepared 3-4 days before eggs and larvae were added to the mixture.

The only rearing formulation which failed to provide minimal requirements for larval development and adult emergence was that of the forest leaf litter infusion. A few adults were reared in the wood chip infusion, but this was unsatisfactory due to fungal growths on the wood chips and unknown chemicals associated with the wood. Natural breeding substrates associated with banana trees and cocoa pods are presently being evaluated as potential laboratory rearing mediums. Midge rearing data from these

materials indicate that sufficient food substances are available for development of the immature stages. Rice hull infusion is also being evaluated as a primary rearing media. Colonization data evaluating rice hulls as a rearing substrate are presented in Table 1.

Presently, little is known about the nutritional requirements for successful larval development. Therefore, the availability and types of possible food substances found associated with the laboratory experimental medias and natural field breeding sites are being studied. It is hoped that new or improved media will result from these studies.

**Pupation and Adult Emergence.** Pupae of C. paraensis were observed to determine the behavior and physical requirements for pupation and adult emergence. Present findings indicate that mature larvae will pupate when submerged in a aqueous medium or contained on a saturated substrate. Immature midge pupae lack buoyancy and are incapable of swimming movements as observed with pupae of mosquitoes. Lateral movements are accomplished by a series of abdomen-tail thrusts. Observations of mature larval activities prior to pupating have not revealed a definable behavior pattern of larvae searching for preferred pupation sites. Preliminary data on pupation time indicate that the pupal stage of C. paraensis last from 2 to 5 days at  $26.5 \pm 1^{\circ}\text{C}$ .

Within 12-18 hrs after the 4th instar larvae pupate, the pupae can ascend to the water surface by means of a physical buoyancy. The mode of ascent is believed to be the result of a gaseous accumulation in the ventral cephalothorax (Figure 2).

When disturbed, the pupae cannot sound (submerge). However, when displaced from the water surface the pupa will quickly float to the surface, and when necessary, it will initiate lateral abdominal movements to properly reorient the respiratory horns with the water-air interface. The pupae stay at the water surface prior to adult emergence approximately 48 to 72 hrs. The adult escapes the pupal exuviae by a longitudinal split along the anterior-dorsal cephalothorax.

**Laboratory Colonization.** Preliminary observations on adult midges obtained by laboratory colonization efforts are as follows:

1. Sex ratio - A 1:1 sex ratio has been obtained under insectary conditions for the  $F_2$  generation (based on account of 715 specimens).

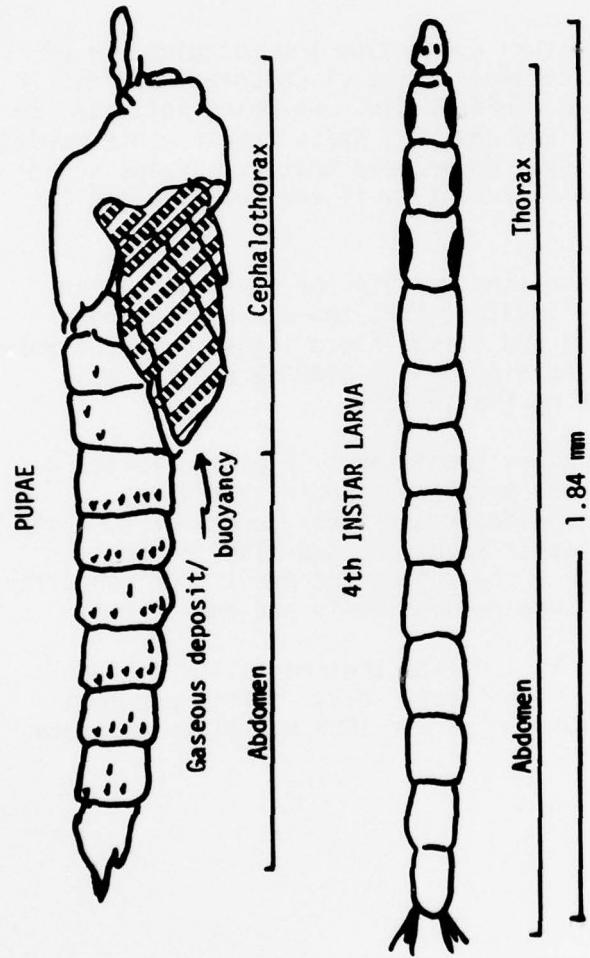


Fig. 2 . Illustrating the pupae and 4th instar of Culicoides paraensis.

2. Mating - Mating of C. paraensis has been observed in laboratory holding cages. Successful fertilization has also been demonstrated by finding sperm bundles in spermathecae dissected from laboratory reared females.

3. Oviposition - Eggs oviposited by female midges reared in the laboratory are being evaluated for viability; however, present observations indicate that a high percent of the eggs are infertile.

One of the most critical factors concerning the colonization of any insect is to obtain successful mating of colonized adults. Consequently, laboratory and field studies are being initiated to determine the environmental and physical space requirements needed to optimize successful mating. We believe that C. paraensis can be successfully reared in the laboratory if the requirements for mating can be established.

Size of Blood Meal. The resulting weights for non-engorged and blood engorged C. paraensis indicate that there was a 74% body weight increase due to blood and tissue fluid ingestion. The mean body weight of a non-engorged midge was 0.0588 mg and the mean blood meal weight was 0.435 mg (Table 2).

Adult Longevity Under Laboratory Conditions. Field collected C. paraensis of unknown ages were maintained under laboratory conditions (26.5°C, 95% RH) to determine their length of survival. Saturated cotton pads (10% sugar solution) and blood donor hamsters were used to maintain the Culicoides until 100% mortality was recorded. Dead midges were removed daily and enumerated.

Preliminary results (Figure 3) indicate that mortality of the study population was low for the first 5 days. Fifty per cent mortality was recorded at 10-11 days and 100% mortality was noted 6 days later.

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Table 1  
Studies on Adult *Culicooides paraensis* (Goeldi) Emergence Under Laboratory Rearing Conditions  
(95% + RH, 26.5°C ± 1°) Using Rice Hulls as a Larval Substrate.

Chamber Number	Egg Oviposition Date	Adult Emergence		Number Emerged	Days From Oviposition to 1st Emergence	Days From Oviposition to 75% Emergence	Days Required for Complete Emergence
		1 <sup>a</sup>	1 <sup>b</sup>				
1	07/05	05/06	29/06	130	29	15	24
2	07/05	03/06	14/07	84	27	31	41
3	23/06	18/07	17/08	179	25	14	29
4	23/06	19/07	15/08	128	26	12	27
5	24/06	29/07	02/09	69	35	19	35
6	29/06	01/08	29/08	75	33	18	28

a Date of 1st emergence  
b Date of last emergence

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Table 2  
Laboratory Observations on the Blood Meal Weights of Culicoides paraensis (Goeldi) Feeding on  
Hamsters, Belém, Pará, Brazil.

Number of Specimens	Total Weight (mg)	Individual Weight (mg)	$\bar{X}$ Weight of Blood Meal (mg)	% Body Weight Increase With Blood Meal
41	2.4100	UNENGORGED		
		ENGORGED	.0588	
42	4.3000		.1023	.0435
				74%

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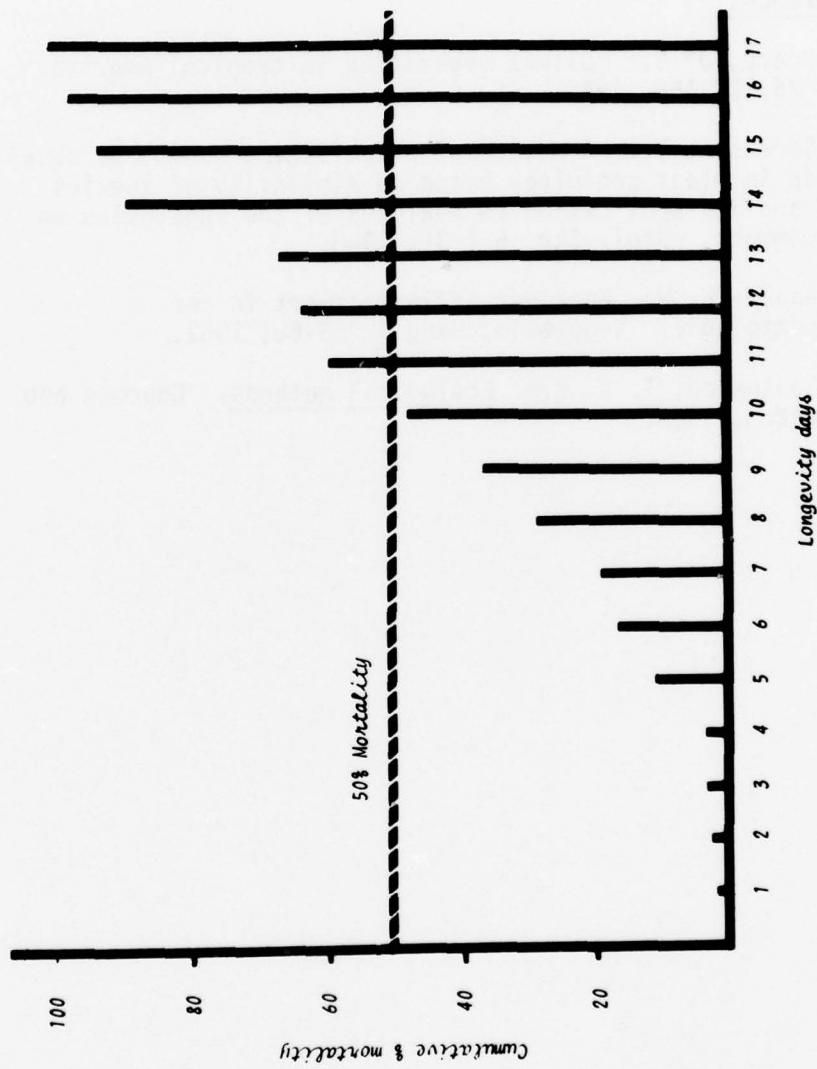


Fig. 3

Longevity of field collected of *Culex quinquefasciatus* under laboratory conditions  
(26.5°C, 95% RH)

PROJECT 3M762770A802 TROPICAL MEDICINE

TASK 00, TROPICAL MEDICINE

Work Unit 010 Disease Transmission in Tropical Populations.

Literature Cited

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION# DA OC 6447	2. DATE OF SUMMARY 77 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
5. DATE PREV SURVY 76 10 01	6. KIND OF SUMMARY D. Change	7. SUMMARY SCRTY U	8. WORK SECURITY U	9. REGRADING NA	10. DISCHG INSTRN NL	11. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: a. PRIMARY 62770A	PROGRAM ELEMENT 3M762770A802	PROJECT NUMBER 00	11. LEVEL OF SUM- A. WORK UNIT 011	TASK AREA NUMBER	WORK UNIT NUMBER	
b. CONTRIBUTING	c. CONTRIBUTING CARDS 114F					
12. TITLE (Precede with Security Classification Code) (U) Health Care and Management of Laboratory Animals						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS 010100 Microbiology						
13. START DATE 76 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House			
17. CONTRACT/GRANT	18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS	20. FUNDS (in thousands)			
a. DATES/EFFECTIVE: NA	PRECEDING 77	CURRENT 2	284			
b. NUMBER: c. TYPE: d. AMOUNT:	FISCAL YEAR 78	2.5	386			
e. KIND OF AWARD: f. CUM. AMT.						
19. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research Washington, DC 20012 ADDRESS:	20. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Division of Veterinary Resources ADDRESS: Washington, DC 20012					
RESPONSIBLE INDIVIDUAL NAME: Raptmund, Garrison, COL, MC TELEPHONE: 202 576-3551	PRINCIPAL INVESTIGATOR (Punish SEAN // U.S. Academic Institution) NAME: LTC Thomas J. Keefe, DVM TELEPHONE: 202 427-5370 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]					
21. GENERAL USE Foreign Intelligence not considered	ASSOCIATE INVESTIGATORS NAME: Marvin Rogul, Ph.D. NAME: James Brendle, B.S.					
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Disease surveillance; (U) Klebsiella pneumonia; (U) Aotus; (U) Random-source dogs; (U) Viral Respiratory Disease; (U) ELISA						
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) To investigate diseases and/or conditions affecting laboratory animals used specifically for military research to enhance production quality and health management and to provide research animals free of known or potential pathogens. The ability to provide clinical diagnosis to laboratory animal problems peculiar to the WRAIR animal colonies is critical to the specific research conducted by the WRAIR. The establishment of a disease data storage/retrieval system will provide unique epidemiological information not available from any other laboratory source. 24. (U) Conventional epidemiological, pathological, and microbiological methods are employed; unconventional procedures are developed as needed. 25. (U) 76 10 - 77 09 A disease surveillance program for laboratory animals was designed and implemented as compatible with automatic data processing. Klebsiella pneumonia was recovered from subhuman primates, rodents, dogs and cats, and was associated with bacteremia and/or respiratory disease in the Aotus. Cytomegalo-like viruses were recovered from 12 of 44 of the Aotus tested. The aerobic and anaerobic intestinal flora of mystromys, which have been affected with fatal diarrhea, was qualitatively and quantitatively defined. An epizootiological study of severe respiratory disease in random-source dogs suggested that procurement of dogs immune to canine distemper would significantly reduce disease. Seroassay of dogs immediately after arrival substantiated this hypothesis. An enzyme-linked immunosorbant seroassay was successfully developed to detect antibodies vs canine adenoviruses 1 and 2, and shows promise for use with canine distemper. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.						

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\*Available to contractors upon originator's approval.

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Project 3M762770A802 MILITARY ANIMAL RESOURCES DEVELOPMENT

Task 00 Military Animal Resources Development

Work Unit 011 Health Care and Management of Laboratory Animals

**Investigators:**

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L. N. Binn, Ph.D; R. L. Marchwicki, B.S.; J. Alford, CPT, VC;  
R. Thomas, PFC; C. Osmola, SP4; M. Riley, PFC; L. Palmer, PFC;  
A. Robinson, SP4; R. Von der Porten, PFC.

**Description:**

Investigate diseases and/or conditions affecting laboratory animals used specifically for military research to enhance production quality and health management and to provide research animals free of known or potential pathogens.

During the reporting period, research activities have included the: (1) design and implementation of a disease surveillance program compatible with automatic data processing, (2) definition and control of respiratory disease in random-source dogs, and (3) development and evaluation of an enzyme-linked immunosorbant seroassay.

1. Design and implementation of a disease surveillance program.

Laboratory animals used at the WRAIR for medical research are either colonized or purchased commercially. Any comprehensive program addressing the health care and management of these animals must necessarily include these interrelated functions: (1) disease surveillance and prevention, (2) disease investigation and control, and (3) research. Colonized animals (mice, rats, guinea pigs, some cats and monkeys) must be periodically examined to detect the presence of colony-threatening infection, as well as to define their microbial flora. The ability of commercially obtained animals to survive quarantine and research holding in a healthy state must be assessed. Disease episodes must be examined in a systematic and standardized manner permitting retrospective epizootiological investigation. Appropriate data must be stored in a readily retrievable manner, providing an institutional memory. At the beginning of the reporting period, none of these desiderata existed. Since then, considerable progress has been made in (a) designing and implementing a disease surveillance program, (b) designing laboratory forms and selecting computer software compatible with epizootiological requirements.

The appropriate sample sizes for bacterial surveillance of "closed" breeding colonies of mice, rats, and guinea pigs were based on their total annual population, the assumption being that periodic samples are cumulative with time, and the desire to detect within 90% confidence an organism present in approximately 20% of the animals. The proximate S-4 Inspection level of military standard 105 D with sample sizes between G and H for tightened inspection was selected to approximate this sampling level. Accordingly 7 mice, 5 rats, and 4 guinea pigs were selected at 6-week intervals for bacterial evaluation. Larger sample sizes were used for the less expensive serological examination. Bacterial examinations were standardized using tissues of the respiratory and intestinal tract as the likely sources for pathogens with epizootic potential and for comparison with tissues submitted for diagnostic purposes. Tissues were preserved in 10% buffered formalin for subsequent histopathological examination. Serological examinations were conducted by a commercial laboratory to detect colony threatening viral infections and to monitor levels of environmental contamination. The mouse antigens are: PVM, Reo 3, GD7, Ectromelia, Sendai, mouse adenovirus, MHV, LCM, and K virus. The rat antigens are: Kilham virus, rat coronavirus, PVM, Sendai, and LCM. The guinea pig antigens are: PVM, SV5, Sendai, and LCM. Open, discontinuous populations, represented by rabbits, cats, dogs, and subhuman primates, are periodically added to by shipments from commercial suppliers. Consequently, periodic sampling cannot accurately "profile" these populations. Presently, all commercially obtained animals are examined for ecto- and endo parasites. Also, all dogs are serologically examined on arrival for the presence of microfilaria of Dirofilaria sp. and for susceptibility to severe respiratory disease.

Qualitative aerobic and anaerobic bacteriology of the trachea, lung and mesenteric lymph node, and quantitative aerobic bacteriology of the duodenum and cecum have been conducted since September 1976 on colonized mice, rats, and guinea pigs. Results obtained from animals periodically submitted for quality control define the expected relative frequency, stability and ratio of the bacterial flora. The flora of "healthy" animals provide a "yardstick" against which to evaluate flora in animals of the same colony when sick. The presence and/or amount of opportunistic bacteria can be measured over time to evaluate the effectiveness of "barriers" in colony operations. Table 1, Bacteriology Surveillance Report for May 1977, illustrates the type and amount of bacteria found in selected tissues of colonized guinea pigs. Note the lung and cranial mesenteric lymph node were sterile as well as duodenal contents when diluted 1:100 and plated on enteric media. Bacteria detected in the trachea, mostly Gram + aerobes and anaerobes, and cecum were, with one exception, expected non-pathogenic flora. The Klebsiella pneumonia and Pseudomonas aeruginosa are opportunists, with usually a low morbidity for rodents. The bacterial monitoring of guinea pigs, mice, and rats was particularly directed at the pathogens or opportunists

shown in Table 2, and were always reported regardless of the specimen from which recovered. Additionally, any bacterium isolated from expectedly sterile tissues, e.g. lungs, lymph node, was reported. Selected specimens were submitted for laboratory examination during the course of disease investigations conducted by personnel of the Departments of Veterinary Pathology or Animal Resources.

Bacteria recovered from "healthy" animals on routine surveillance and suspected as being pathogens or opportunists were compared with those recovered from selected tissues of moribund animals (Table 3). Sporadic deaths associated with otitis affected C3H mice during October, 1976. The mice were purchased commercially and placed in animal rooms unequipped with water chlorinators. P. aeruginosa was the pathogen. On surveillance, P. aeruginosa was recovered in low numbers (approximately  $10^3$ /gram cecal contents) from 10 of 35 colonized mice. K. pneumonia was recovered from the trachea of 2 of these mice. Only one sick rat was submitted for examination. Corynebacterium kutscheri, a known pathogen, was isolated from a leg abscess of this rat. On surveillance, K. pneumonia was recovered from an intestinal abscess of 1 rat, and from the respiratory tract and/or gut of 3 other rats. C. kutscheri was recovered from the lung of one rat on surveillance, and 7 had P. aeruginosa as high as  $10^8$ /gram of cecal contents. Unhealthy guinea pigs were submitted for examination more frequently than any other rodent. On three occasions, K. pneumonia and/or B-hemolytic streptococci were recovered from the throat or lung of animals with pneumonia. The surveillance bacteriology of rodents was an unqualified success in detecting those pathogens or opportunists most likely to be encountered diagnostically. The absence of water chlorinators in building 40, and the frequent breakdown of chlorinators in building 512 was detected and/or emphasized by the prevalence and amount of P. aeruginosa. The association of K. pneumonia with abscesses and/or pneumonia in rats and guinea pigs appeared related to the higher prevalence of the bacterium in these colonies as compared with the mice.

The seroassay of mice, rats and guinea pigs for detection of selected viral infections disclosed no frank pathogens. Rats were usually infected with PVM, Sendai, and Kilham rat virus, but only 2 of 10 had antibodies vs rat coronavirus. Seroassay of only 7 guinea pigs showed all infected with PVM, and none with SV5 or Sendai. Comparing serological results obtained from mice in room 3 vs mice in room 6 showed some interesting dynamics of viral infection. The spotty, focal nature of PVM infection was evident by the markedly higher antibody prevalence rate among mice of room 6 (40%) vs that of mice in room 3 (10%). Through March 1977, mice in both rooms showed consistently high prevalence rates to Sendai virus. From April through June 1977, Sendai infections of mice in rooms 3 and 6 have been virtually absent. Apparently, virus transmission was blocked in the colony by the transfer of maternal antibody to enough susceptible young.

Diagnostic specimens from dogs, cats, and rabbits were received for bacteriological examination. Most specimens from 24 sick or dead dogs were received in conjunction with viral respiratory disease. The most prevalent pathogen and/or opportunists isolated from these dogs were Bordatella bronchiseptica (10) B. hemolytic streptococci (6) and Staphylococcus aureus (5). K. pneumonia was also isolated. B. hemolytic streptococci and K. pneumonia were isolated from 7 sick cats and 1 dead cat with respiratory disease and/or conjunctivitis. Pasteurella multocida and B. bronchiseptica were the most frequent pathogens covered from 4 sick and 5 dead rabbits affected with "snuffles."

The African white-tailed rat, Mystromys albicaudatus, was recently added to the ranks of laboratory animals at the WRAIR. The animals are colonized at the AFIP, but on one occasion a group was housed in a room at the WRAIR alongside rats and mice. Some of the latter mystromys acquired fatal diarrhea. No frank bacterial pathogens were isolated from the contents of the duodenum, cecum, or feces. Consequently, the intestinal flora of 5 "healthy" mystromys were examined quantitatively, aerobically and anaerobically from duodenum, cecum, and feces.

Anaerobes were detected in only 3 of 5 animals, probably because the lack of a glove box precluded strict anaerobic handling of tissues. Also, the speciation of the nonpathogenic coryneform and Bacillus required special procedures which were impractical to perform. In the duodenum, aerobic Gram + organisms were consistently observed as were staphylococci-micrococci, Bacillus sp., Coryneform sp., and occasionally yeast. Anaerobically, Gram + rods (Lactobacillus sp.) were also consistently observed. Gram - bacteria, aerobic or anaerobic, were not observed in the duodenum. In the cecum, Gram - and Gram + aerobic flora were common. Bacillus sp. were always present, as were molds or yeasts. Occasionally, Escherichia coli and P. aeruginosa were isolated. Anaerobically, Gram + rods (Lactobacillus sp.) and cocci (Gaffkya sp.) predominated with occasional Gram - rods (Bacteroides sp.). The fecal flora were similar in type and number to those identified from the cecum.

This evaluation indicated first that the intestinal microbial flora of mystromys from the duodenum to the feces always contained Gram + flora, and usually fungi. Aerobic Gram + rods (Bacillus sp.) and cocci (staphylococci-micrococci), and anaerobic Gram + rods (Lactobacillus sp.) predominated. Second, Gram - bacteria, aerobic or anaerobic, were not found in the duodenum, but were often present in the cecum and feces. E. coli and P. aeruginosa were inconsistently present in the cecum and feces, and in relatively low numbers (> 10 /ml). Third, recovery of "normal" anaerobic

flora could not be accomplished adequately without use of a glove box or similar apparatus. Finally, identification of nonpathogenic coryneform or *Bacillus* bacteria beyond the Genus level was not practical.

Respiratory disease was a major problem affecting nonhuman primates during the past year. Pneumonia killed 4 baboons. *S. pneumoniae* and *K. pneumoniae* were recovered from pneumonic tissue of two baboons each. The throat of another baboon, successfully treated for pneumonia during April 1977, yielded heavy growth of *K. pneumoniae*. Finally, over a 3 day period in December 1976, the baboon colony experienced an epizootic of upper respiratory disease. Nasal swabs obtained from 14 baboons, most of which were coughing and sneezing, yielded *B. bronchiseptica* and *K. pneumonia* as suspect pathogens.

Glomerulonephritis, Gram - bacterial pneumonia and/or septicemia, air sacculitis, and anemia were the multisystemic diseases affecting many of the colonized *Aotus* monkeys in building 511 at the WRAIR during the past year. Tissues from 31 dead *Aotus* were submitted for bacterial and viral isolation. Tissues from the following number of monkeys were infected with the corresponding bacterium: *K. pneumonia* - 11, *Streptococcus* sp. - 5, *Proteus* sp. - 4, *Staphylococcus aureus* - 4, *P. aeruginosa* - 3, *B. bronchiseptica* - 1, and *E. coli* - 1. Many animals showed histopathological lesions of glomerulonephritis. Throat specimens from the following numbers of monkeys, judged clinically ill, yielded moderate to heavy growth with the corresponding bacterium: *K. pneumonia* - 15, *S. aureus* - 5, *Proteus* sp. - 4, *P. aeruginosa* - 3, mixed culture - 10.

During the past year, virus studies were initiated to detect and evaluate viruses from *Aotus* monkeys with renal and/or respiratory diseases. Procedures for the isolation and identification of viruses were similar to those used for dogs except that an owl monkey kidney cell line was used. This cell line has a broad host range for viruses of the *Aotus* monkey. Isolates were grouped by type of cytopathic effect (CPE) for subsequent identification. In addition, kidney organ cell cultures were prepared from 2 monkeys with renal disease and subpassaged with trypsin for at least 15 passages. Viruses were recovered from the throat and rectal specimens of sick or "healthy" monkeys but not from the lungs, livers, spleens, and kidneys of dead monkeys. Based on the type of CPE, the isolates have been placed into 3 groups. The first isolates were from the throat and rectal specimens of a monkey with generalized herpesvirus disease. The latter viruses produced an identical herpes-like CPE which rapidly spread throughout the cell culture. Intranuclear eosinophilic inclusions were evident in H&E stained infected cultures. The CPE was similar to that produced by Herpesviruses simplex and tamarinus previously isolated from *Aotus* monkeys at the WRAIR. Studies to identify these isolates are in progress.

The second group of cytopathic agents were recovered only from throat specimens of "normal" and sick monkeys. The first and reference isolate, 7933Thr, was detected 12 days after inoculation. A focal area of rounded cells was seen which gradually spread to adjacent cells. The affected cells detached from the glass surface leaving a hole in the cell sheet. Agents producing similar CPE have been detected up to 24 days post inoculation. Passage of the reference isolate produced CPE earlier depending on the virus dose. The slow spread of the CPE suggested that the agent was cell associated. Separate titrations of cell and supernatant fractions from infected cultures confirmed cell association in that 100-fold higher titers were present in the cell fraction. Large intranuclear inclusions were evident in H&E stained infected cell cultures. Examination of infected cell cultures in the electron microscope was done in collaboration with CPT J. Seely, VC, (Department of Veterinary Pathology, WRAIR). Mature viral particles were observed within the nucleus and cytoplasm and were typical of herpesviruses. The intranuclear forms had either a naked nucleocapsid surrounding an inner nucleoid ring or a naked nucleocapsid with an electron dense ring. The average diameter of the nucleocapsid was  $104.5 \pm 2.5$  nm, the inner nucleoid ring was  $66. \pm 5.0$  nm. The inner core measured  $55 \times 30$  nm. The intracytoplasmic virions were enveloped and larger than the intranuclear forms. The virions often were evident in vacuoles associated with electron dense granules. These findings are consistent with the cytomegaloviruses of the herpesvirus group. Agents producing CPE similar to the 7933Thr, were recovered only from throat specimens of 8 other apparently healthy and 3 sick monkeys (Table 5). Similar viruses were reisolated from 5 of these monkeys after an interval of several months. These findings suggest that these viruses (presumably cytomegaloviruses) are frequently present and persist in the throats of the Aotus monkeys. Further studies on the identification and distribution of these CML-V are in progress.

The third agent which produced a characteristic type of CPE was recovered from the rectal specimen of a monkey with renal disease. Rounded cells were seen in the culture and progressed throughout the cell monolayer. On subculture the CPE appeared on the sixth day and progressed to involve approximately 25% of the cells by the eleventh day. Intranuclear inclusions also were evident in infected cell cultures with this agent (8138R). These inclusions were distinct from those observed with the previously described agents. Further work is in progress to identify this agent.

In summary, Gram negative enteric bacteria, principally K. pneumonia, have been predominately associated with Aotus sick with air sacculites, or those which have died with pneumonia or sepsis. Current research is directed towards ascertaining whether or not K. pneumonia is also casually related to the anemia and glomerulonephritis. In addition, viral agents producing 3 distinct types of CPE have been recovered from "normal" Aotus and those

sick with glomerulonephritis and/or respiratory disease. Cytomegalo-like agents were found in the throats of approximately 25% of the monkeys tested and could be reisolated after several months. These viruses may produce persistent infections in Aotus. Current research is directed towards identifying the viruses and evaluating their role in the multisystemic disease of Aotus. It is noteworthy that cytomegaloviruses are frequently recovered from the throat, urine, and blood of other species of monkeys. The presence of persistent viremias in Aotus could modify their response to experimental infection with malarial parasites.

Attempts to obtain historical, epizootiological information on the natural history of diseases affecting different animal colonies at the WRAIR have been largely unsuccessful. An analysis of research needs and service functions was made to define the requirements for automatic data processing. The primary aim of computerization was to assemble pertinent knowledge from clinical, laboratory, pathology, and management sources, and disseminate the accumulated knowledge in such a way to improve the health care and management of laboratory animals. Two computerized systems available at the WRAIR were evaluated for their ability to store and selectively retrieve and collate microbiological/epizootiological data. The FAMULUS program, basically a library sort system, was too cumbersome for manipulating microbiological data. The QUIS (Query Update Information System) program appeared suitable.

All information was to proceed through channels illustrated by the flow diagrams in Figures 1a, and 1b, coordinating through the bacteriology section of the Department of Clinical Investigation and Research. Information derived from sick, healthy surveyed, and necropsied animals will be entered onto a specimen information sheet. Figure 2 illustrates the latest specimen information sheet. This sheet has the bacteriology accession number, pathology ID number and animal ID number, and links the information of all three services. A spelling/synonym dictionary has been written so that the information submitted will be standardized for such fields as "Requestor, Institute, Division, Department, Animal, Scientific name, bacterial name etc.,." Standardization assures that "canine," "K-9," and "dog" will all be entered as "dog," and equated. The sheet is designed so that the left side requires only a simple entry for some fields, such as a checkmark. For instance the "sex" field entries require only a check except for the sex field subfield "Other, Specify." The appropriate query would be, "list accession numbers where sex subfield equal "other"." The written contents will either be automatically printed or a response will be made to "see comments." The sheet is designed to accommodate epidemiological, specimen and animal characteristics, and reasons for specimen submission, e.g., routine or special surveys, necropsies, experimentation, etc. This is the link between "Normal" animals and their flora compared to diseased animals and their flora. Another major field is "Animal Under Treatment," which may qualify the amount and types of bacteria isolated.

The "presumptive diagnosis" field is subject to the greatest editing and interpretation. Accordingly, the left side is a laboratory code which describes the organ and function system which best describes the diagnosis and specimens. For instance, throat and fecal samples sent in for diagnosis with a presumptive diagnosis of renal disease would both be coded as "urogenital, kidney." The "procedures requested" field directs the laboratory to conduct specific procedures usually compatible with confirming the presumptive diagnosis.

The program has a text scanning capacity which can be used in the "history" or "Comments" field.

The bacteriology laboratory has three functions which are intimately linked to the submission sheet. Aerobic and anaerobic bacteriology also include fungi and mycoplasma functions. Each of the functions has a laboratory work sheet.

The aerobic work sheet (Table 6) accommodates five isolates per specimen. The accession number is assigned to the specimen. The reactions used to identify isolated bacteria are recorded on this worksheet. Responses to each field are usually confined to + or -. When these responses are inadequate, "Grow" fields are available which further describe the organism on additional media. The entries are part of a syllabus that can be continuously edited. The resulting identification is standardized and listed under "Laboratory diagnosis". Quantitation of bacteria is listed under counts, and their relative numbers are listed as the number of quarters of a petri dish which are occupied by the bacteria. For example, on the same dish S. aureus may occupy 4 quarters, S. fecalis 2 quarters and E. coli 1 quarter. The organism of predominance is S. aureus. Antibiotic sensitivities are also listed by its Kirby-Bauer zone size and corresponding interpretation of sensitivity or resistance. The laboratory worksheets for anaerobic (Table 7) bacteria and mycoplasma (Table 8) are designed in a similar manner.

A preliminary mockup of the anaerobic laboratory with entries into the OUIS program demonstrated that many correlations were easily obtained. The program holds promise for deriving a great number of extrapolations over a period of time. Predictions of disease trends and trends of antibiotic resistance and sensitivity appear attainable in the near future.

In summary, computer systems available to the WRAIR were evaluated by their capabilities for storing, collating, and retrieving microbiological data for research, epizootiological, and quality control purposes. A series of laboratory work sheets were designed to accommodate these data. These sheets interface with the clinician and pathologist. On preliminary evaluation, a system called QUIS appears promising for meeting all requirements. Some of its special features are english oriented commands, key words, data maintenance, direct sequential or generic searching, text scanning, encode/decode, field editing, and Boolean and relational comparisons.

## 2. Definition and control of respiratory disease in random-source dogs.

In December 1975, a prospective study was started to define the viral etiology of respiratory disease in unconditioned random source dogs (RSD). Parainfluenza SV5 and the Toronto A26/61 adenovirus were isolated frequently from sick dogs and the minute virus of canines (MVC) and canine herpesvirus rarely were recovered. In addition an unidentified virus was isolated from the lungs of a dog with fatal respiratory disease. Although canine distemper virus (CDV) was not recovered, clinical, histopathological and serological evidence clearly indicated that this virus was the most important cause of prolonged severe respiratory diseases. (WRAIR, Annual Reports 1975-1976). This report summarizes further virus isolations on post-mortem tissues from dogs with fatal respiratory disease and additional serological tests to define the incidence of MVC and canine coronavirus infections in sick dogs.

Virus isolations were attempted from throat and rectal swab specimens obtained at the onset of clinical signs and after death from tissues of 9 dogs with fatal respiratory disease (Table 9). Histopathologic examination of tissues from 7 dogs clearly indicated that each also were infected with CDV. Three or more viruses were recovered from fatal cases and in several instances the same virus was reisolated after an interval of 9 to 20 days. The Toronto A26/61 virus and SV5 were recovered most frequently from both the swabs and post-mortem tissues of the respiratory tract. SV5 was isolated also from the liver, kidney and spleen of 2 dogs. Canine herpesvirus was isolated from nasal or throat specimens of 3 dogs but only once from the trachea. Coronaviruses were recovered from the intestinal mucosa of 3 dogs and from the lungs of a fourth dog. The latter isolate was the first recovery of a coronavirus from canine lung. Each of these coronaviruses were neutralized by guinea pig antiserum vs canine coronavirus strain 1-71.

Serological tests were done to define the incidence in sick dogs after arrival, of MVC and canine coronavirus infections (Table 10). Increased antibody titers to the MVC occurred in almost all shipment groups of dogs, with an overall incidence of 23%. Canine coronavirus infections occurred at a similar rate, 21%, with members of 3 of 6 shipment groups having increased titers. These viruses appeared to be more communicable than canine herpesvirus but significantly less communicable than SV5.

These findings confirmed and extended those reported last year (WRAIR Annual Report 1975-1976). Unconditioned RSD were infected with numerous viruses, often concurrently, which produced epizootics of respiratory disease. The dogs susceptible to infection with CDV were often infected with several agents. Consequently these dogs had prolonged illnesses and a high mortality rate. In addition to CDV, Toronto A26/61 virus and SV5 were consistently present and were highly communicable.

Virus studies to define the etiology of respiratory disease in unconditioned random source dogs were completed. Virus isolation tests and histopathologic examination of tissues from fatal infections provided evidence that multiple viral infections in these dogs were frequent events. Inclusions of CDV were evident in each of the dead dogs examined, and the Toronto A26/61 canine adenovirus and SV5 were frequently recovered. In addition canine coronaviruses were isolated from the intestinal mucosa of 3 dogs and the lungs of a fourth. The latter isolate was the first recovery of a canine coronavirus from the lung of a dog. Serological studies indicated the incidence of infections among newly arrived dogs with both canine coronavirus and the MVC was approximately 20%. The findings clearly indicated the need to procure dogs immune to CDV, and canine adenoviruses in order to control epizootics of debilitating and/or fatal respiratory disease in RSD.

An epizootiological study was designed to evaluate the effect of pre-existing immunity to CDV and ICH virus on: (1) reducing the prevalence and severity of respiratory disease; (2) reducing the expense of issuing healthy laboratory dogs; and (3) increasing the capacity of this facility to issue healthy laboratory dogs. In addition, data are presented showing the importance of monitoring each group of dogs on arrival for CD antibody.

A total of 66 conditioned and 68 unconditioned dogs from at least 3 different and entire shipments by the same vendor were used for comparing the relative cost from receipt through quarantine. More than 95% of the conditioned dogs met the serological requirements for CD and infectious canine hepatitis (ICH) described below. An additional 195 conditioned dogs from a second vendor were evaluated serologically vis a vis their subsequent respiratory disease.

New dogs were quarantined in 5 rooms, each containing 15 runs measuring 4' by 10'. Dogs were housed individually within runs. Dogs from each shipment were maintained as discrete populations within separate rooms. A sixth room that contained 14 cages and 8 runs was used to house dogs under treatment to convalesce from known or suspected transmissible diseases. When this room was filled, ill dogs were kept and treated in their quarantine room. Under these circumstances, the entire shipment remained in quarantine until every dog in the group was healthy.

All R-S, unconditioned dogs were held a minimum of 30 days for quarantine and conditioning, whereas all conditioned dogs were held for 14 days. Upon arrival, each dog was given a physical examination, and specimens were obtained for hematological, serological, and fecal examinations. Each dog was vaccinated on day 2 against CD, ICH, leptospirosis,<sup>a</sup>

<sup>a</sup> Tissuevax 5, Pitman Moore, Washington Crossing, NJ, 2.0 ml, subcut.

and rabies.<sup>b</sup> Dogs were treated with either ampicillin<sup>c</sup> or tetracycline<sup>d</sup> daily for 7 days starting on day 3 after arrival. Each was wormed with dichlorvos<sup>e</sup> on days 3 and 12, and with niclosamide<sup>f</sup> on day 13. An electrocardiogram and a thoracic radiograph were obtained and a second physical examination was given at the end of the quarantine period.

Blood was collected from all R-S dogs within 24 hours after arrival. Sera were obtained from blood specimens and stored at -20C. Microtiter techniques were used to obtain SN antibody titers to CD, ICH, and canine parainfluenza virus (SV5) viruses. Dogs having CD titers of  $\geq 1:100$  and ICH titers of  $\geq 1:20$  were considered immune to these viruses. Dogs whose sera showed titers vs SV5  $\geq 1:2$  were considered seropositive.

Per diem charges were calculated by dividing the sum of attendants' salaries, and the cost of feed supplies and overhead by the average daily dog population. During the study, an average daily population of 234 dogs cost \$1.33 per dog per diem, distributed as \$1.06 for labor, \$0.15 for food and supplies, and \$0.12 for overhead.

Drug and vaccine expenses for the unconditioned dogs were obtained by dividing the average monthly pharmacy expense (\$836) for October through December, 1976, by the average monthly population of quarantined dogs (241) during that period. Drug and vaccine costs for conditioned dogs were obtained similarly using the average monthly pharmacy expense (\$572) for January through March, 1977, as the base, and an average monthly quarantine population of 200. Clinical pathology expenses averaged \$5.00 per dog.

<sup>b</sup> Endural-R, Norden Laboratories, Lincoln, NB, 1.0 ml, I.M.

<sup>c</sup> Ampicillin, Bristol Laboratory, Syracuse, NY, 10 mgm/lb, 3 times daily, orally.

<sup>d</sup> Tetracycline HCL, Rochelle Lab., Long Beach, CA, 10 mgm/lb, 3 times daily, orally.

<sup>e</sup> Dichlorvos, Agriculture Division, Shell Chemical Co., San Ramona, CA, 13.6 mgm/lb, orally.

<sup>f</sup> Niclosamide, Haver-Lockhart Laboratory, Div Bayvet Corp., Shawnee, KA, 70.0 mgm/lb, orally.

Clinical respiratory disease developed after arrival in 42 of the 68 (62%) unconditioned dogs which were evaluated for cost analysis. Nineteen (28%) of the ill dogs died, and 21 (31%) required an extension of the quarantine period, with a median of 52 days (Table 11). The onset of respiratory disease began as early as 2 days and as late as 22 days after arrival. Tissues from 5 of 19 dead dogs were examined histologically, and all 5 had histological lesions of CD. By contrast, only 2 of the 66 (3%) conditioned dogs which were evaluated for cost analysis developed mild respiratory disease during the abbreviated 14-day quarantine period, and none died. All but 3 (98%) of these dogs had protective levels of SN antibody to CD virus on arrival. Each of the 66 conditioned dogs was considered to be healthy, and was available for issue after the 14-day quarantine period.

A comparison of quarantine costs for unconditioned vs conditioned dogs is shown (Table 12). The purchase price for the unconditioned dogs was 25% lower than for conditioned dogs. However, the issue cost of a conditioned dog was 40% less than an unconditioned dog. The projected annual occupancy for conditioned and unconditioned dogs (Table 13) was based on the theoretical receipt and issue of conditioned dogs every 14 days, and unconditioned dogs every 52 days. All reasons that back to back shipments are not likely apply equally to both groups, and were discounted for comparison purposes. By purchasing conditioned dogs, almost twice the projected requirement of 1200 dogs could be issued annually, whereas only 34% of this requirement could be met with unconditioned dogs. The projected monetary savings for the WRAIR associated with the purchase of properly conditioned dogs exceeds \$64,000 per year.

The almost total absence of respiratory disease in the 66 conditioned dogs that had protective titers to CD and ICH on arrival greatly exceeded expectations and prompted further investigation. Larger groups of conditioned (195) and unconditioned (158) dogs were examined serologically to evaluate whether or not CD vaccination alone was responsible for the reduction in disease incidence (Table 14). The 66 conditioned dogs described above had > 95% antibody prevalence rates on arrival to the highly communicable CD, ICH, and SV5 viruses. Use of combined ICH and CD vaccines was the probable reason for the usual equivalent prevalence rates of CD and ICH antibodies. Expectedly, the 158 unconditioned dogs, which did not have to be vaccinated or held by the vendor more than 5 days before shipment,<sup>1</sup> serologically showed substantial susceptibility to these viruses on arrival. Most (74%) of the unconditioned dogs that developed respiratory disease were either susceptible to or incubating CD virus on arrival. However, as much as 23% of the respiratory disease cases occurred in dogs with protective levels of SN antibody on arrival vs CD virus (Table 15). The group of 195 conditioned dogs obtained from a second vendor (B) showed markedly lower antibody prevalence rates to CD, ICH, and SV5 on arrival than did

the group of 66 conditioned dogs from vendor A. Respiratory disease occurred in 9 of the 45 (20%) conditioned dogs from vendor B which arrived without serological evidence of prior CD vaccination, and 3 died of CD. Additionally, SV5, ICH, and canine herpesvirus infections were detected during the quarantine of these conditioned dogs from vendor B by virus isolation and serological techniques.

In summary before 1976, attempts to prevent severe respiratory disease in R-S dogs by contractual requirements before arrival in accordance with the ILAR specifications and by vaccination and therapy after receipt usually were unsuccessful (unpublished data). Results from an epizootiologic study suggested that prevention of CD alone would substantially reduce morbidity and mortality among R-S dogs. Development and utilization of rapid SN tests for detection of CD and ICH antibodies permitted evaluation of dog populations which were > 95% immune to CD and ICH upon arrival. The effect of these immunological criteria, which were additional to those already stipulated for a conditioned dog, was more startling than expected. None of the dogs died, and respiratory disease was minimal. Further investigation revealed that the reduction in the morbidity and mortality associated with respiratory disease among the immunologically defined conditioned dogs could not be exclusively attributed to vaccination against CD and ICH. Dogs which were held 30 days or more by the vendor after vaccination and before shipment not only arrived immune to CD and ICH, but had also naturally acquired immunity to SV5. Conversely, when other populations of conditioned dogs (vendor B) arrived with antibody prevalence rates > 80% to CD, there was an increased occurrence of respiratory disease, particularly in dogs without CD antibody. More than 60% (28 of 45) of the latter dogs from vendor B arrived susceptible to SV5, indicating an incomplete conditioning period. Dogs incubating fatal CD infections will not develop protective titers vs CD. These observations clearly show the importance of assuring the presence of SN titers vs CD for each dog upon arrival. Herd immunity was quite effective in preventing respiratory disease when < 95% of the newly arrived dogs were immune to CD. Herd immunity was only marginally effective in preventing respiratory disease when 77% of the dogs on arrival showed immune titers to CD. Sero-assays were not only essential in predicting the fate of individual dogs to CD after arrival, but were valuable for prognosticating respiratory disease in general for the entire group. Since Schroeder et al<sup>14</sup> have shown that the clinical course of CD is not altered if exposure to CD virus occurred more than 24 hours before vaccination, vendors should vaccinate all prospective R-S dogs against CD and ICH immediately upon arrival at their kennels. This procedure should reduce the vendor's losses as substantially as it has the WRAIR's.

By purchasing conditioned, R-S dogs which were immune on arrival to CD and ICH, the WRAIR was able to reduce by more than 1 month the time needed for preparing a healthy laboratory dog for issue. This reduction in quarantine time has increased the availability of issuable laboratory dogs by more than 100% annually, and has reduced their average cost by 40%.

The present critical shortage of R-S dogs for laboratory use can be substantially reduced, along with unnecessary canine suffering, if CD vaccinations were done before or at the earliest time after R-S dogs are brought into contact with other dogs. Additionally, institutions largely can prevent respiratory disease among newly arrived R-S dogs by selecting only those dogs which have SN titers of  $\geq$  1:100 vs CD virus.

### 3. Development and evaluation of an enzyme-linked immunosorbant assay.

In an effort to support the production of healthy research animals the usefulness of an enzyme-linked immunosorbant assay, ELISA, has been investigated as a rapid screening test for detection of antibodies to antigens of clinical importance in veterinary microbiology.<sup>5</sup> The ELISA is a newly developed immunological test which offers many advantages over current methods. It is a rapid, environmentally safe and relatively uncomplicated assay. The reagents have a long shelf-life and yield objective results with equal or greater sensitivity than radioimmunoassay (RIA), in some systems.<sup>6</sup> ELISA is adaptable for field use because it is not restricted to a central work area and disposal facility as are the RIA and serum neutralization test, SN. It is a biochemical rather than a biological assay, therefore concern about contaminating cell cultures is eliminated. Compared to the SN the ELISA is also less time consuming and labor intensive due to the elimination of the continuous requirement for tissue culture cells.

Previous seroassays of random source dogs by SN test indicated that dogs with high antibody titers to canine distemper virus (CDV), and infectious canine hepatitis virus (ICHV) had less respiratory illness during their stay at the WRAIR than dogs without pre-existing antibodies to these viruses. By accepting only those dogs which possess antibody titers to these viruses, treatment costs and conditioning time can be kept to a minimum. Therefore, the ELISA was developed to screen for antibodies to these viruses.

CDV was propagated in Vero cells within 420 x 100 mm roller bottles. Initially the Vero cells tended to clump and form dense bands of cell growth around the bottle resulting in uneven monolayers. Special cleaning procedures for the roller bottles eliminated the clumping and banding of cells. The roller bottles were cleaned with concentrated NaOH (70g/100ml H<sub>2</sub>O) and rinsed 3 times with tap water then twice with deionized water.

Each roller bottle was inoculated with approximately  $10^8$  cells which were usually obtained from the harvest of 3, 32 oz prescription bottles or one half of a roller bottle. The trypsinized cells were disrupted by aspiration through a 14 guage canula and syringe about 14-15 times and then were suspended in 100 ml of Minimal Essential Medium, (MEM), containing 10% fetal bovine sera, 1% glutamine and 1% antibiotics. Each roller bottle containing 100 ml of suspended cells was incubated at 37/C and rotated<sup>a</sup> for 15 minutes at 60-90 rph. The pH was adjusted to approximately 7.2 by gassing with CO<sub>2</sub> for 10-15 seconds, followed by continued rotation at 60-90 rph for an additional 45 minutes. The rotation was then reduced to 30 rph. At one hour intervals for the next 5-6 hours the roller bottles were shaken vigorously for 5 seconds in an upright position to obtain more uniform dispersion and attachment of cells. After 5-6 hours the medium containing unattached cells was replaced with 200 ml of fresh medium. The roller bottles were incubated until monolayer confluence was reached in about 5-7 days. Additional medium replacement was usually not necessary. Occasionally, the medium was replaced on the sixth day after seeding if the monolayer was not confluent.

Vero cell monolayers in roller bottles were infected using 15 ml of the Onderste poort strain of CDV which titered between 10<sup>4.0</sup> and 10<sup>5.0</sup> per ml. The inoculum was allowed to adsorb for 1 hour 37/C at a rotation of 30 rph before adding 50 ml of fresh medium. About 16 hours before harvest the medium was replaced with 50 ml of MEM without serum. At 42 hours post-inoculum the medium from 4 roller bottles was placed into a 250 ml centrifuge bottle and centrifuged at 300 x g for 10 minutes. The pellet was resuspended in approximately 2.0 ml of serum-free medium and put into one of the roller bottles containing infected cells without medium. The supernatant from each of the infected monolayers was saved for titration. Next, 15 ml of serum-free medium was added to each roller bottle. These were then freeze/thawed 3 times in rapid sequence using an alcohol-dry ice bath. The detached cells were collected and placed into a 50 ml conical centrifuge tube. An additional 5 ml of serum-free medium was used to rinse the remaining cells from each roller bottle. The cell suspension was chilled in an ice bath and sonicated in 30-40 ml aliquots at a setting of 7 using the micro tip of a sonicator for 30 seconds.<sup>b</sup> The sonicate was then centrifuged at 300 x g for 10-15 minutes. The supernatant was placed in 3 ml vials, frozen in an alcohol-dry ice bath and stored at -60C.

<sup>a</sup> Cell Production Roller Apparatus Model 7300, Bellco Glass, Inc.  
Vineland, NJ.

<sup>b</sup> Ultrasonics Cell Disrupter Model W185, Plainview, L.I. NY.

The Cornell strain of ICHV and Toronto virus strain C955 were propagated in monolayers of primary dog kidney cells (PDK) within 32 oz bottles. Uninfected monolayers were rinsed 3 times with 10 ml of M-199 Earle's BSS medium with 1% antibiotics. Each monolayer was infected with 4.0 ml of a virus suspension titering  $10^{4.5}$  per ml. After stationary adsorption for 2 hours at 37°C the inoculum was replaced with 50 ml of serum-free medium. Virus was harvested when cytopathic effect was observed on 50% or more of the monolayer. These were freeze/thawed 3 times in rapid sequence using an alcohol dry ice bath. The suspensions were clarified by centrifugation at 300 x g for 15 minutes. The supernatant was stored in 3 ml vials at -60°C for titration and use as antigen.

Standard anti-CDV serum and standard anti-ICHV serum were obtained from USDA, VBL, NADL, Ames Iowa 50010 and stored at -20°C. Homologous anti-ICHV sera were obtained by immunizing 3 seronegative dogs with ICHV vaccine. Homologous Toronto virus sera were obtained by infecting 2 seronegative dogs with Toronto virus. Serum samples were collected on days 7, 14, 21, 28, 35, and 42 post infection and sera were stored at -20°C. These sera were used to demonstrate the sequential development of antibody with the micro ELISA.

Rabbit anti-dog IgG conjugated to horseradish peroxidase<sup>c</sup> was used in the ELISA for detection of canine IgG. The conjugate was stored at -20°C and was diluted 1:2000 with PBS-Tween buffer containing 1% bovine serum albumin immediately prior to use.

The substrate solution was a mixture of 5-amino salicylic acid and hydrogen peroxide.<sup>8</sup>

The polystyrene micro ELISA substrate plates were used for all ELISA.<sup>d</sup> CoStar microtiter plates were used for micro serum neutralization (SN) test<sup>d</sup>.

The following sequential steps briefly describe the micro ELISA. The microsubstrate plate was coated with antigen by adding 0.1 ml of optimal antigen dilution per well, and dried overnight 37°C. Plates were washed 3 times, 3 minutes each, with PBS-Tween buffer. Sera were added 0.1 ml per well, and the mixture was incubated 30 minutes 37°C. Plates were washed 3 times, 3 minutes each with PBS-Tween. Distilled water containing 1% bovine serum albumin (BSA) was added, 0.1 ml per well, and this mixture was incubated 30 minutes 37°C. The BSA bound any active groups of polystyrene which had not been coated with antigen or antibody. Plates were washed 3 times, 3 minutes each with PBS-Tween.

<sup>c</sup> Miles Laboratories Inc., Elkhart, IN.

<sup>d</sup> Cooke Laboratories, Alexandria, VA.

Conjugate was added, 0.1 ml per well, and incubated 30 minutes 37 C. Plates were washed 3 times, 3 minutes each with PBS-Tween. Substrate solution was added 0.1 ml per well, and incubated 1 hour at room temperature. The color reaction was read visually or by a spectrophotometer. Visually the color was scored 4+, 3+, 2+, 1+, - with a 1+ considered a positive reaction. Controls for the test were antigen, conjugate, and positive and negative sera. Optimal concentrations for specificity and sensitivity were obtained for each "lot" of antigen and conjugate.<sup>9</sup>

The following sequential steps briefly describe the procedure for the micro SN test. Sera were inactivated for 30 minutes 56C. Serial two-fold dilutions in 50  $\mu$ l volumes were made across the microtiter plate. A virus suspension titering 100 times its endpoint was added, 50  $\mu$ l per well. Plates were incubated for 1 hour room temperature. Cells (240,000 per ml) were added to each well in 0.1 ml amounts. Plates were incubated 3 days/37C in CO<sub>2</sub> atmosphere. Cytopathic effect was read. Cell control wells contained no virus.

Detection of CDV antibody by micro ELISA has been difficult because the CDV is tightly cell-associated.<sup>10</sup> The cell membranes present in the antigen preparations were associated with non-specific binding of conjugate. Attempts to purify and concentrate the preparations<sup>11</sup> resulted in increased nonspecific reactions. Figure 3a shows the results of box titrations of several recent CDV preparations. The antigen control wells of the neat antigen preparation in each case show a positive color reaction due to non-specific membrane binding of the conjugate. This background color can be reduced by diluting antigen preparation, thus reducing the concentration of cell membranes present. Technical aspects of the micro ELISA for detection of antibody to CDV have advanced to where results can now be compared with those obtained by SN test.

The ICH and Toronto viruses are not highly cell-associated but are released into the surrounding medium. Virus suspensions obtained from nutrient media rather than the cell pack were highly specific when used as antigens, without the complication of nonspecific reactions caused by the binding of the conjugate by cell membranes. The box titration in Figure 3b illustrates this point. The sequential development of homologous and heterologous antibodies in dogs which were experimentally infected with either ICHV or Toronto virus was evaluated using both the ELISA and SN test, Figures 4 and 5. The preinoculation sera were all negative. Antibody was detected as early as 7 days in some sera by both assays. The levels of antibody detected were similar using both tests, indicating that the ELISA measured the same antibody as the SN test. The ELISA was also sensitive enough to demonstrate the group antigen shared by ICHV and Toronto virus. Gross reacting antibody evoked by heterologous antigen were detected in all 5 sera using both the ELISA and the SN test.

Sera were collected from 65 random-source dogs immediately after their arrival at the WRAIR and examined by both ELISA and micro SN test as a further evaluation of the sensitivity and specificity of the ELISA. Table 16 shows the results of two methods of comparing the ELISA to the micro SN test. There was a 98.5% agreement between the two assays. The ELISA had a sensitivity (ability to call a positive serum positive) of 98% and a specificity (ability to call a negative serum negative) of 100%.

The ELISA has been successfully developed to detect canine antibody against ICHV. It can detect similar levels of neutralizing antibody as the SN test and possesses a high degree of agreement, sensitivity and specificity when compared to the SN test. Research to develop the ELISA to detect canine antibody against CDV will continue.

Project 3M762770A802 MILITARY ANIMAL RESOURCES DEVELOPMENT

Task 00 Military Animal Resources Development

Work Unit 011 Health Care and Management of Laboratory Animals

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Table 1. Bacteriology surveillance report

	16 May 1977		
	Routine Guinea Pig Bacteriology Survey		
	Bldg 512, Room #5		
	3 Female Breeders		
	1	2	3
LUNG	-	-	-
TREA	Bacillus sp. Neisseria sp. Alpha hemolytic streptococci Peptococcus sp.	-	-
MLN	-	-	-
DUO <sup>c</sup>	-	-	-
CEC	Enterococci (2) <sup>b</sup> K. pneumoniae (2) Peptostreptococcus intermedius	Enterococci (4) K. pneumoniae (2) E. coli (2) Peptostreptococcus intermedius	E. coli (2) Peptostreptococcus intermedius

<sup>a</sup>Unidentified anaerobes. Anaerobes are not quantified.

<sup>b</sup>( ) = log 10 dilutions.

<sup>c</sup>Duodenum examined at  $10^{-4}$  dilutions on MacConkey agar for aerobic bacteriology; contents are not diluted for anaerobic bacteriology.

Bacterial examinations were performed on lungs, trachea (TREA), mesenteric lymph node (MLN), duodenum (DUO), and cecum (CEC).

Table 2. Bacterial pathogens and opportunists of the mouse, rat, and guinea pig.

	Guinea pig	Rat	Mouse
<u>Salmonella</u> sp.	X	X	X
<u>Bordetella bronchiseptica</u>	X	X	X
<u>Streptococcus pneumoniae</u>	X	X	X
<u>Pyogenic streptococci</u>	X	X	X
<u>Klebsiella pneumoniae</u>	X	X	X
<u>Yersinia pseudotuberculosis</u>	X	X	X
<u>Streptobacillus moniliformis</u>	X	X	X
<u>Pasteurella pneumotropica</u>		X	X
<u>Pasteurella multocida</u>		X	X
<u>Corynebacterium kutscheri</u>		X	X
<u>Pseudomonas aeruginosa</u>		X	X
<u>Mycoplasma</u> sp.		X	X

**Table 3.** Bacterial pathogens or opportunists recovered from rodents on surveillance and moribund rodents from Sept 1966 to Sept 1977.

Species and month	Surveillance Bacterium	Infection Rate	Moribund animals		
			Presumptive diagnosis	Bacterium	Tissue
<u>Mouse</u>					
Sep 76	<u>Pseudomonas aeruginosa</u>	5/7			
Oct 76		0	Otitis	<u>P. aeruginosa</u>	Inner ear
Nov 76	<u>P. aeruginosa</u>	2/7			
Apr 77	<u>Klebsiella pneumonia</u>	2/7			
	<u>P. aeruginosa</u>	3/7			
<u>Rat</u>					
Nov 76	<u>Corynebacterium kutscheri</u>	1/5			
Jan 77			Abscess	<u>C. kutscheri</u>	Lung, leg abscesses
Mar 77	<u>P. aeruginosa</u>	4/5			
	anaerobe (lung)	2/5			
May 77	<u>K. pneumonia</u>	3/5			
Jun 77	<u>P. aeruginosa</u>	3/5			
	<u>K. pneumonia</u>	1/5			
<u>Guinea pig</u>					
Oct 76					
Nov 76	<u>K. pneumonia</u>	1/3	Diarrhea	Mixed gram neg.	Duodenum
Dec 76	<u>K. pneumonia</u>	1/3			
Jan 77					
Mar 77			Neck abscess	<u>B. hemol.</u>	Lung Abscess
				<u>streptococcus</u>	
				<u>K. pneumonia</u>	
				<u>B. fragilis</u>	
Apr 77	<u>K. pneumonia</u>	1/3			
May 77	<u>K. pneumonia</u>	2/3			
June 77	<u>B. melanogenicus</u>	1/4	Pneumonia	<u>K. pneumonia</u>	Throat
Aug 77	<u>K. pneumonia</u>	1/4			
			Pneumonia	<u>B. hemol.</u>	Throat
				<u>streptococcus</u>	

1. Isolated from intestinal abscess.
  2. Mice obtained from commercial source.

Table 4. Antibody prevalence rates in retired breeder mice of building 512 to selected viral antigens from November 1976 to June 1977.

Location	No. positive/No. tested (%)			
	PUM	MUM	Sendai	MHU
Room 3	3/30 (10)	4/13 (31)	6/13 (46) <sup>2</sup> 0/37 (0) <sup>3</sup>	9/48 (19)
Room 6	14/35 (40)	8/25 (32)	22/25 (88) <sup>2</sup> 2/39 (5) <sup>3</sup>	5/50 (10)

1. After the first 40 sera were non-reactive, assays for the following antigen were discontinued: Reovirus 3, GD-7, K virus, Polyoma, Ectromelia, mouse adenovirus, and LCM.
2. Prevalence rates through March 1977.
3. Prevalence rates after March 1977.

Table 5. Cytomegalo-like viruses recovered from apparently healthy and sick Aotus monkeys in Bldg 511 and 40 of the WRAIR.

Clinical Findings	Location	No. monkeys with agent/ total examined in specimen	
		Throat (%)	Rectum
Renal disease	511	2/8	0/6
Cervical abscess	511	1 <sup>a</sup> /1	0/1
"Healthy"	511	7 <sup>b</sup> /20	0/5
	40	<u>2/15</u>	<u>0/15</u>
Totals		12/44 (27)	0/27

<sup>a</sup>Reisolated

<sup>b</sup>Reisolated from each of 4 monkeys tested.

Table 6. Laboratory worksheet for aerobic bacteria.

ACCESSION

	1	2	3	4	5	ONPG		1	2	3	4	5
AERO						GEL						
MAC						NIT						
SS						CET						
BGA						TEMP						
GRAM						°C						
STAIN						RXN						
RXN						DNASE						
MORPH						BAP						
CAT						RYN						
OXY						CHOC						
TSI-S						PIG						
B						COAG						
H						OPTI						
G												
IND						BACA						
MR						BEA						
VP						ESC						
MOT						NaCl						
CIT						%						
UREA						RYN						
LYS						SERO						
ARG						PHAGE						
ORN						XFAC						
PHEN						VFAC						
MALO						CO2						
H2S						SERUM						
10%						STRCH						
LAC						CAS						
GLLU						ANIML						
GLU						GROW						
LAC						MED						
SUC						COL						
MAN						RXN						
DUL						TEX						
SAL						SIZE						
ADO						MED						
INO						COL						
SOR						RXN						
ARA						TEX						
RAF						SIZE						
RHA						MED						
XYL						COL						
SYST						RXN						
NAME						TEX						
RXN						SIZE						

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Table 6: Laboratory worksheet for aerobic bacteria (con't).

NO GROW	1	2	3	4	5	NOTES
MED						
MED						
MED						
SENSITIVITIES	1	2	3	4	5	
AM						
B						
CF						
C						
CL						
GM						
N						
NF						DIRECTIONS
P						
PB						
DS						
SSS						
TE						
LAB DIAG	COUNTS					QUARTERS
1.						
2.						
3.						
4.						
5.						

Table 7. Laboratory worksheet for anaerobic bacteria.

Accession # .

Date received

Date reported

Organisms #	Isolation media	1.	2.	3.	4.	5.
Colonial description--						
Gram stain morphology						
Growth anaerobic						
--CO <sub>2</sub>						
--aerobic						
Agar deep--O <sub>2</sub> tolerance						
--reduction						
--gas production						
Motility						
Cooked meat--digestion						
Milk						
Thioglycollate--speed						
--appearance						
Gas chromatography--THR + PROP						
--LAC + PROP						
--by-products	1.					
	2.					
	3.					
	4.					
	5.					
	6.					
	7.					
Antibiotic sensitivities--						
Cephalin (CF)						
Chloromycetin (C)						
Clindomycin (CC)						
Colistin (CL)						
Erythromycin (E)						
Gentamycin (GM)						
Kanamycin (K)						
Lincomycin (L)						
Penicillin G (P)						
Rifampin (RA)						
Tetracyclin (TE)						
Vancomycin (VA)						

Table 7. Laboratory worksheet for anaerobic bacteria. (cont'd)

Organism #	1	2	3	4	5
Enzymes--lecithinase (LEC)					
--lipase (LIP)					
--catalase (CAT)					
--urease (URE)					
Hydrolysis--gelatin (GEL)					
--starch (STH)					
--esculin (ESC)					
Reduction--nitrate (NO <sub>3</sub> )					
Production--indol (IND)					
--H <sub>2</sub> S (H <sub>2</sub> S)					
Carbohydrates--amygdalin (AMG)					
--arabinose (ARB)					
--cellobiose (CEL)					
--fructose(levulose) (FRU)					
--glucose (dextrose) (DEX)					
--glycerol (GLY)					
--inositol (INO)					
--lactose (LAC)					
--maltose (MAL)					
--mannitol (MAT)					
--mannose (MAN)					
--melezitose (MLZ)					
--melibose (MLB)					
--raffinose (RAF)					
--rhamnose (RHM)					
--salicin (SAL)					
--sorbitol (SOR)					
--starch (STA)					
--sucrose (SUC)					
--trehalose (TRE)					
--xylose (XYL)					

Laboratory diagnosis 1.

2.

3.

4.

5.

1010

Comments:

Table 8. Laboratory work sheet for mycoplasmas.

Accession#

MYCOPLASMAS

Date received:

Date reported:

	1.	2.	3.	Notes
<u>Sterol req.</u>				
<u>Glucose</u>				
<u>Arginine</u>				
<u>Isol.</u>				
<u>Atm.</u>				
<u>O or F</u>				
<u>Film</u>				
<u>Spot</u>				
<u>Tzol</u>				
<u>Urea</u>				
<u>Serol.</u>				
<u>Inhib.</u>				
<u>FA</u>				
Name 1.				
2.				
3.				

**Table 9: Viral and histopathological studies of fatal respiratory infections in unconditioned random source dogs.**

Dog No.	Day onset Day died	Virus isolated	(day)	Specimen(s)	Histopathological diagnosis
65908	13/28	SV5 Toronto Coronavirus	{13} (13) (28)	Nose, throat Rectum Lungs	Canine distemper <sup>1</sup>
66123	13 and 33/47	Toronto A26/61 Minute virus can. SV5 SV5	{13} (33) (33) (47)	Throat, rectum Rectum Nose, throat Nose, Throat trachea, lungs pul. lym. node	Canine distemper
27882	14/23	C. herpes	(14,23)	Nose	Canine distemper
66402	16/30	C. herpes	(30)	Nose, throat, trachea	Not done
67268	Unk/16	Toronto A216/61 SV5	{16} (16)	Rectum Nose, trachea, lung, liver Small intestine	Canine distemper
67305	6/23	Coronavirus Toronto A26/61 Toronto A26/61 SV5 C. herpes Coronavirus	(16) {6} (23) (23) (23) (23)	Rectum Trachea Nose, trachea Nose Small intestine	Canine distemper

1. Canine distemper inclusions present in post-mortem inclusions.

Table 9 (con't) Viral and histopathological studies of fatal respiratory infections in unconditioned random source dogs.

Dog No.	<u>Day onset</u> <u>Day died</u>	Virus isolated	(day)	Specimen(s)	Histopathological diagnosis <sup>1</sup>
68300	6 and 16/23	Toronto A26/61 SV5 SV5	{ 6 { 6 (23)	Throat Nose Liver, kidney, spleen Small intestine	Canine distemper <sup>1</sup>
		Coronavirus	(23)		
69379	14/34	Toronto A26/61 Toronto A26/61	{ 14 { 34	Eye Lung	Canine distemper
63258	11/22	Toronto A26/61	(11)	Throat	Not done

1. Canine distemper inclusions present in post mortem tissues.

Table 10. Virus antibody studies of unconditioned random-source dogs with respiratory disease.

Date arrived	No. dogs with increased antibody titer/ total tested (%) to	
	Minute virus of canines (HI) <sup>1</sup>	Canine coronavirus (SN) <sup>2</sup>
18 Dec 75	3/11	3/8
29 Dec 75	1/2	0/2
25 Feb 76	5/19	5/19
11 Mar 76	0/1	0/1
31 Mar 76	1/14	3/22
14 Apr 76	1/1	0/1
TOTALS:	11/48 (23)	11/53 (21)

1. HI = Hemagglutination-inhibition test.

2. SN = Serum neutralization test.

Table 11. Effect of respiratory disease (RD) on the quarantine of random-source conditioned vs unconditioned dogs.

	Unconditioned dogs	Conditioned dogs
No. received	68	66
No. (%) with RD	42 (62)	2 (3) <sup>1</sup>
No. (%) dead	19 (28)	0
No. (%) cleared after routine quarantine	39 (57) <sup>2</sup>	66 (100) <sup>3</sup>
No. (%) with extended quarantine <sup>4</sup>	21 (31)	0
Range (median) in days of extended quarantine	4 to 100 (22)	NA <sup>5</sup>

1. Nasal discharge only.
2. 30 days
3. 14 days.
4. Extended quarantine applies to illness-related days for unconditioned dogs beyond the routine 30 day period.
5. NA = not applicable.

Table 12. Actual costs for quarantining 68 unconditioned vs. 66 conditioned random-source dogs.

Expense category	Unconditioned dog	Conditioned dog
Individual purchase price	\$ 38	\$50
\$1.33/day per diem for 1. Routine quarantine 2. Extended quarantine 3. Mortality quarantine	1. \$40.90 @ 30 da. 2. \$13.83 @ 10.4 da. 3. \$13.17 @ 9.9 da.	1. \$18.62 @ 14 da. 2. None 3. None
Dead dog replacement cost <sup>3</sup>	\$14.75	none
Drug costs	\$15.45	\$13.54
No. dogs issued	49	66
Issue cost/dog	\$136.10	\$82.16

1. Extended quarantine is the number of extra quarantine days beyond the routine 30 day period caused by an illness in unconditioned dogs.  $509 \text{ extra days} + 49 \text{ issued dogs} = 10.4 \text{ days}$ .
2. Mortality quarantine is the number of quarantine days for dogs that died during the routine 30 day period.  $485 \text{ extra days} (19 \text{ dead dogs}) + 49 \text{ issued dogs} = 9.9 \text{ days}$ .
3.  $(19 \text{ dead dogs} \times \$38) + 49 \text{ issued dogs}$ .

Table 13. Projected costs for quarantining unconditioned vs conditioned, random-source dogs.

Category	Unconditioned dogs	Conditioned dogs
Quarantine days	52	14
Daily capacity single-run occupancy	80	80
Annual occupancy @ contiguous-turnover	404 <sup>1</sup>	2,080
Cost/dog	\$136.10	\$82.16
Projected cost of 1200 dog requirement	\$163,320	\$98,592
Projected savings with 1200 dogs	NA	\$64,728

1. 561 less 28% mortality = 404 = 34% of annual requirement.

Table 14. Relationship of antibody to canine distemper virus (CDV) and antibody to other canine viruses in random-source (R-S) dogs on arrival.

Type of dog	CDV antibody	No. (%) of dogs	No. (%) of dogs with antibody to	
			ICH <sup>1</sup>	SV5 <sup>1</sup>
Unconditioned vendor A	0	91 (58)	23 (25)	6 (7)
	+	67 (42)	58 (87)	26 (39)
	Total:	158	81 (51)	32 (20)
Conditioned vendor A	0	3 (5)	3 (100)	3 (100)
	+	63 (95)	62 (98)	61 (97)
	Total:	66	65 (99)	64 (97)
Conditioned vendor B	0	45 (23)	34 (76)	17 (38)
	+	150 (77)	144 (96)	106 (71)
	Total:	195	178 (91)	123 (63)

1. Individual percentage figures were calculated from the numbers in the same row of column 3.

Table 14. Relationship of antibody to canine distemper virus (CDV) and antibody to other canine viruses in random-source (R-S) dogs on arrival.

Type of dog	CDV antibody	No. (%) of dogs	No. (%) of dogs with antibody to	
			ICH <sup>1</sup>	SV5 <sup>1</sup>
Unconditioned vendor A	0 +	91 (58) 67 (42) Total: 158	23 (25) 58 (87) 81 (51)	6 (7) 26 (39) 32 (20)
Conditioned vendor A	0 +	3 (5) 63 (95) Total: 66	3 (100) 62 (98) 65 (99)	3 (100) 61 (97) 64 (97)
Conditioned vendor B	0 +	45 (23) 150 (77) Total: 195	34 (76) 144 (96) 178 (91)	17 (38) 106 (71) 123 (63)

1. Individual percentage figures were calculated from the numbers in the same row of column 3.

Table 15. The relationship of pre-existing antibody to canine distemper virus (CDV) and subsequent respiratory disease during the preparation of random-source dogs for laboratory use.

Type of dog	CDV antibody	No. (%) of dogs	No. of dogs with disease		Total affected (%)
			Non-fatal	Fatal	
Unconditioned vendor A	0	91 (58)	37	30	67 (74)
	+	67 (42)	13	3	16 (23)
	Total :	158	50	33	83 (53)
Conditioned vendor A	0	3 (5)	0	0	0 (0)
	+	63 (95)	2	0	2 (3)
	Total:	66	2	0	2 (3)
Conditioned vendor B	0	45 (23)	6	3	9 (20)
	+	150 (77)	8	0	8 (5)
	Total:	195	14	3	17 (9)

**Table 16.** Two methods of comparing serological results obtained by the enzyme-linked immunosorbant assay, ELISA, vs the serum neutralization, SN, test in cells.

Method 1.	Percentage of Agreement Tests		RESULTS
	ELISA	SN	
No. of Sera			
18	18 (-)	18 (-)	
46	46 (+)	46 (+)	64 = Agree
1	1 (-)	1 (+)	1 = Disagree
0	0 (+)	0 (-)	65 Total

$$\text{Percentage of agreement} = \frac{64}{65} = 98.5\%$$

**Method 2.** Sensitivity and Specificity of a Test<sup>1</sup>

ELISA	Serum Neutralization Test		
	Positive	Negative	Total
Positive = 46	(A) 46	(B) 0	46
Negative = 19	(C) 1	(D) 18	19
	(A+C) 47	(B+D) 18	65

$$\text{Sensitivity} = \frac{A}{A+C} = \frac{46}{47} = 97.9\%$$

$$\text{Specificity} = \frac{D}{B+D} = \frac{18}{18} = 100\%$$

1. Sensitivity is defined as the ability to declare a positive serum, positive. Specificity is defined as the ability to call a negative serum, negative.

Figure 1a. Data processing of specimen information.

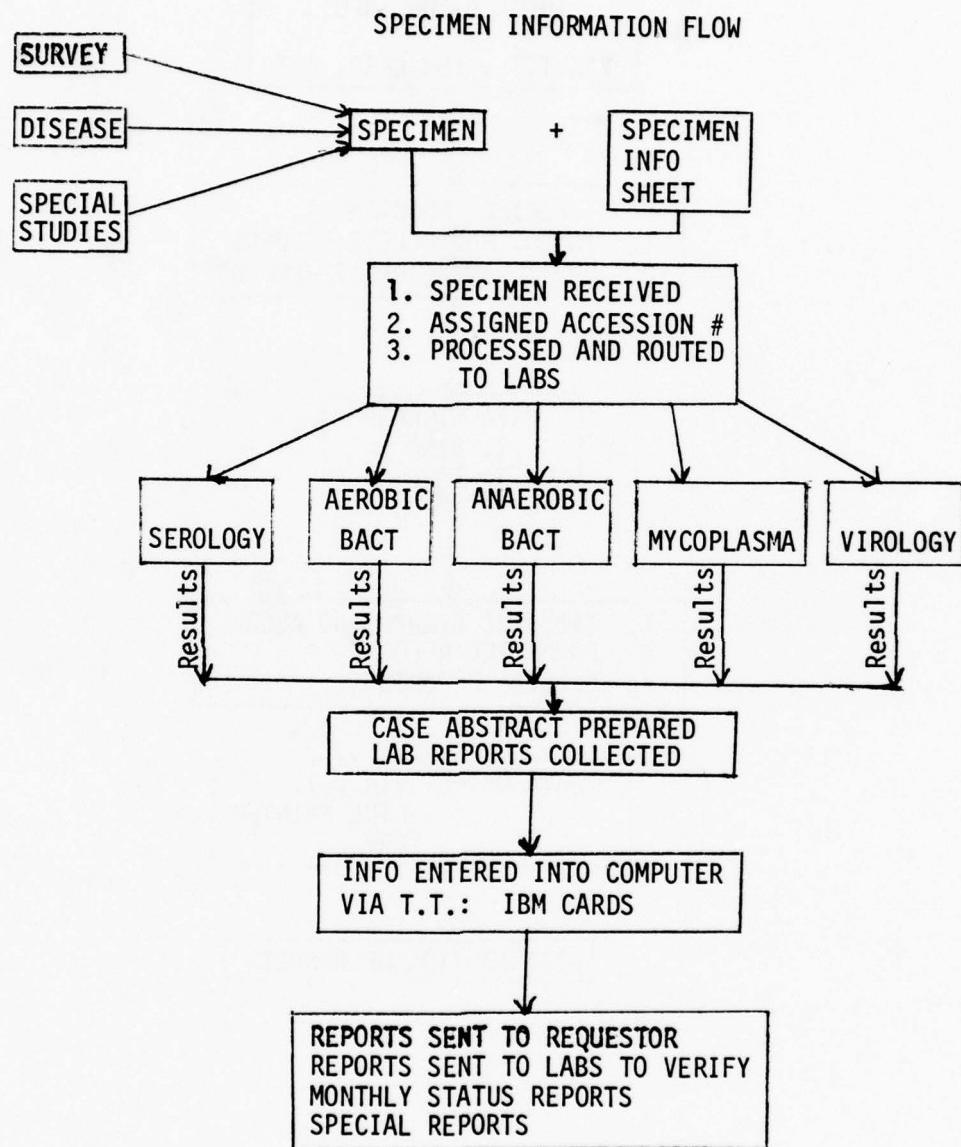


Figure 1b. Data processing of specimen information.

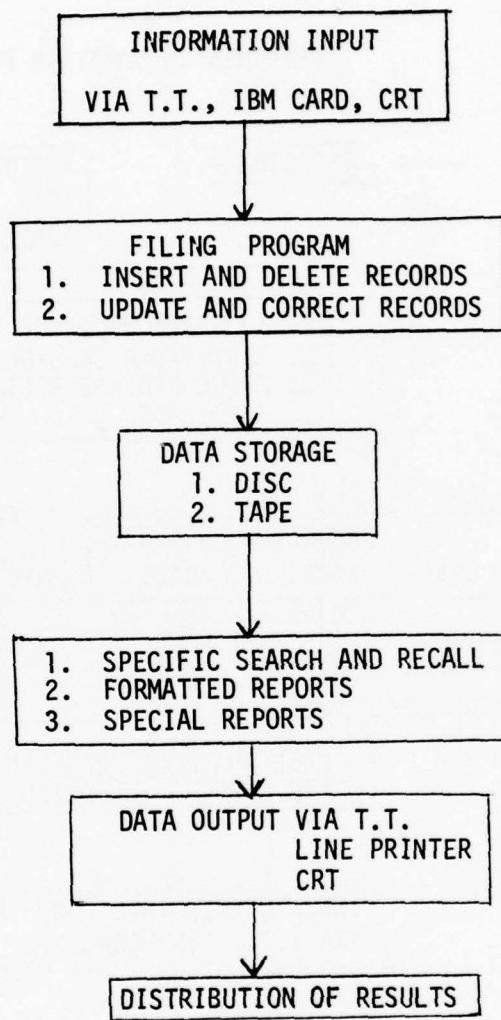


Figure 2. Specimen information sheet.

SPECIMEN INFORMATION

Accession No. \_\_\_\_\_

Pathology ID No. \_\_\_\_\_

DATE: \_\_\_\_\_ Year \_\_\_\_\_ Month \_\_\_\_\_ Day \_\_\_\_\_ REQUESTOR: \_\_\_\_\_

SUBMITTED BY: \_\_\_\_\_ Institute \_\_\_\_\_ Division \_\_\_\_\_ Department \_\_\_\_\_

SPECIES: \_\_\_\_\_ Animal \_\_\_\_\_ Scientific name \_\_\_\_\_

ANIMAL I.D. No. \_\_\_\_\_

SOURCE OF ANIMAL: \_\_\_\_\_ Purchased, from whom? \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
WRAIR Bred  
\_\_\_\_\_  
Other, Specify \_\_\_\_\_

LOCATION: \_\_\_\_\_ Bldg No. \_\_\_\_\_ Room No. \_\_\_\_\_

SEX: \_\_\_\_\_ F  
\_\_\_\_\_  
F-Breeder  
\_\_\_\_\_  
F-Neuter  
\_\_\_\_\_  
M  
\_\_\_\_\_  
M-Neuter  
\_\_\_\_\_  
Other, Specify \_\_\_\_\_

AGE \_\_\_\_\_ Date of Birth: \_\_\_\_\_

WEIGHT: \_\_\_\_\_

SPECIMEN \_\_\_\_\_ Whole Animal  
\_\_\_\_\_  
Tissue, What kind? \_\_\_\_\_  
\_\_\_\_\_  
Excreta. What kind? \_\_\_\_\_  
\_\_\_\_\_  
Culture. Of what: \_\_\_\_\_  
\_\_\_\_\_  
Other, Specify \_\_\_\_\_

Figure 3a. Box titrations of three lots of Ondersteopoort strain of canine distemper virus vs. homologous standard reference serum.

Ag dilution	Lot # 25				Lot # 26				Lot # 27			
	Neat	2.5	5	10	20	Neat	2.5	5	10	20	Neat	2.5
1:10	4	4	3	1		4	4	2	1		4	4
20	4	4	2	4		4	4	3	1		4	4
40	4	4	3	1	+/-	4	4	2	+/-	+/-	4	4
80	4	2	2	+/-	-	4	3	2	+/-	+/-	4	4
160	4	1	1	+/-	-	4	2	1	-	-	4	1
320	4	+/-	+/-	-	-	4	1	+/-	-	-	4	+/-
640	4	+/-	+/-	-	-	4	+/-	-	-	-	4	+/-
Ag Control + 4	-	-	-	-		4	-	-	-		1	-
Ag Control	-	-	-	-							-	-

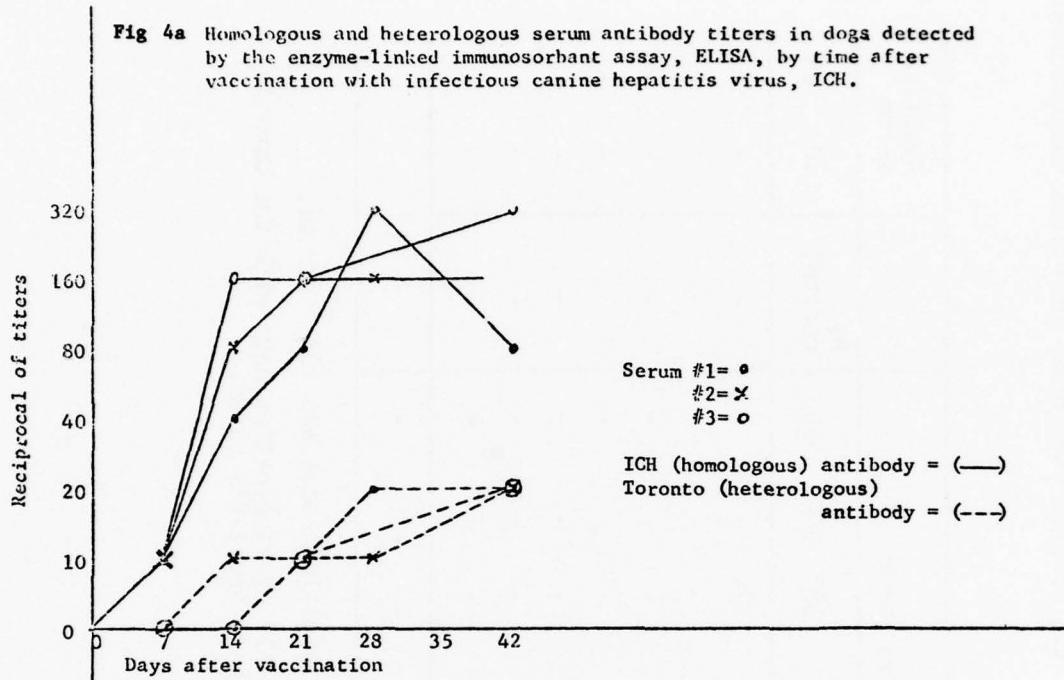
1. USDA, VBL, NADL, Ames, Iowa.
2. The 50% tissue culture infectious dose for lot number 25, 26 and 27 respectively were  $10^{6.0}$  and  $10^{5.5}$  per ml.

Figure 3b. Box titration of one representative lot<sup>1</sup> of the Cornell strain of infectious canine hepatitis virus vs. homologous reference serum<sup>2</sup>.

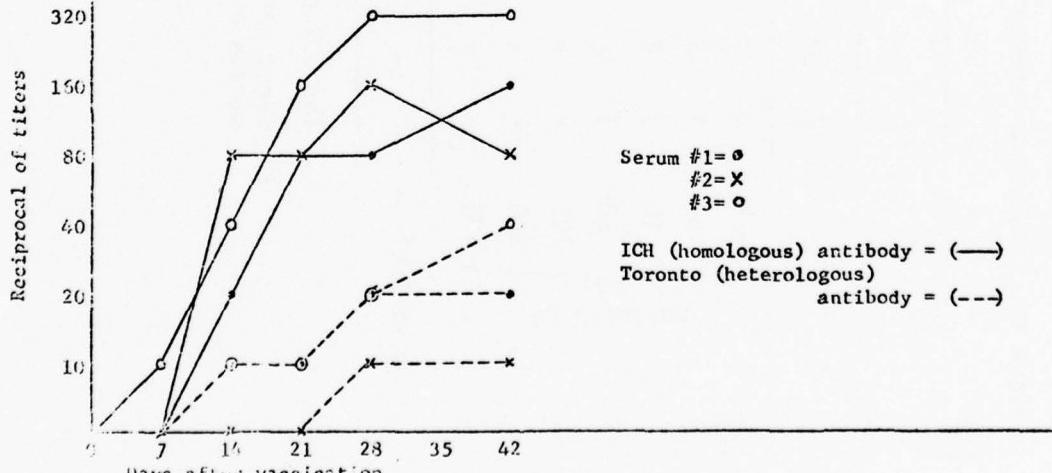
	Positive serum						Negative serum				
	10	20	40	80	160	320	640	1280	Ag control	10	25
Neat	4	4	3	2	1	+/-	-	-	-	-	-
1:5	3	3	2	2	1	-	-	-	-	-	-
10	3	2	1	+/-	-	-	-	-	-	-	-
Ag dilution	50	1	+/-	+/-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-
200	-	-	-	-	-	-	-	-	-	-	-
400	-	-	-	-	-	-	-	-	-	-	-
Ab control	→	-	-	-	-	-	-	-			

1. The 50% tissue culture infectious dose was  $10^{4.2}$  per ml.  
 2. Serum produced at the WRAIR by vaccinating dogs with the Cornell strain of infectious canine hepatitis virus.

**Fig 4a** Homologous and heterologous serum antibody titers in dogs detected by the enzyme-linked immunosorbent assay, ELISA, by time after vaccination with infectious canine hepatitis virus, ICH.

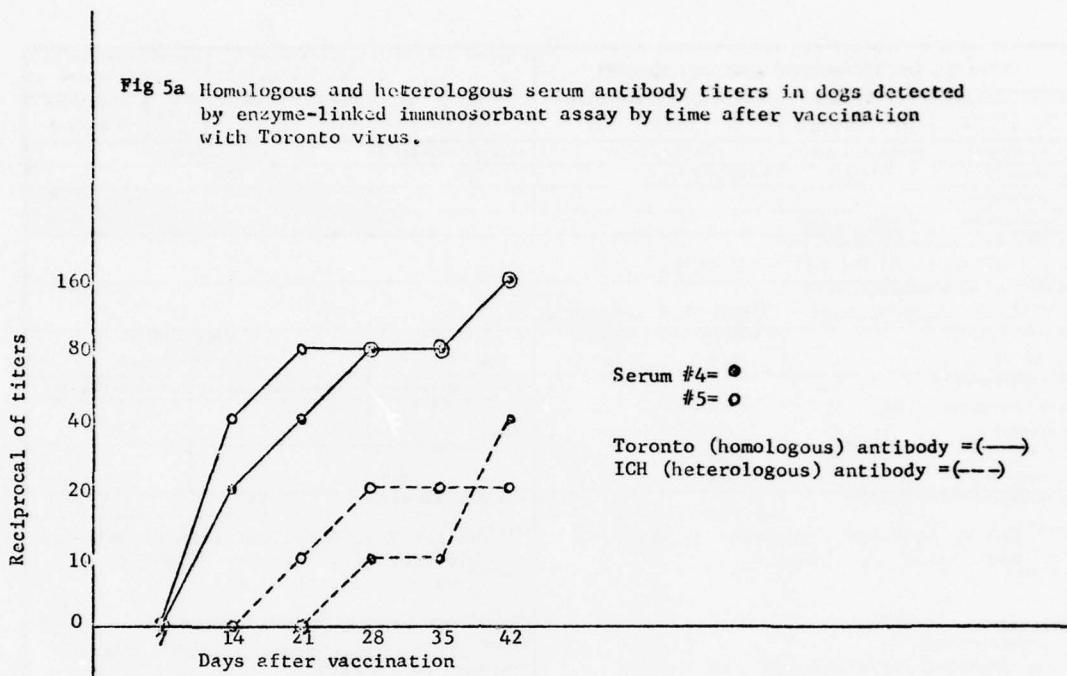


**Fig 4b** Homologous and heterologous serum antibody titers in dogs detected by the neutralization test by time after vaccination with infectious canine hepatitis virus, ICH.

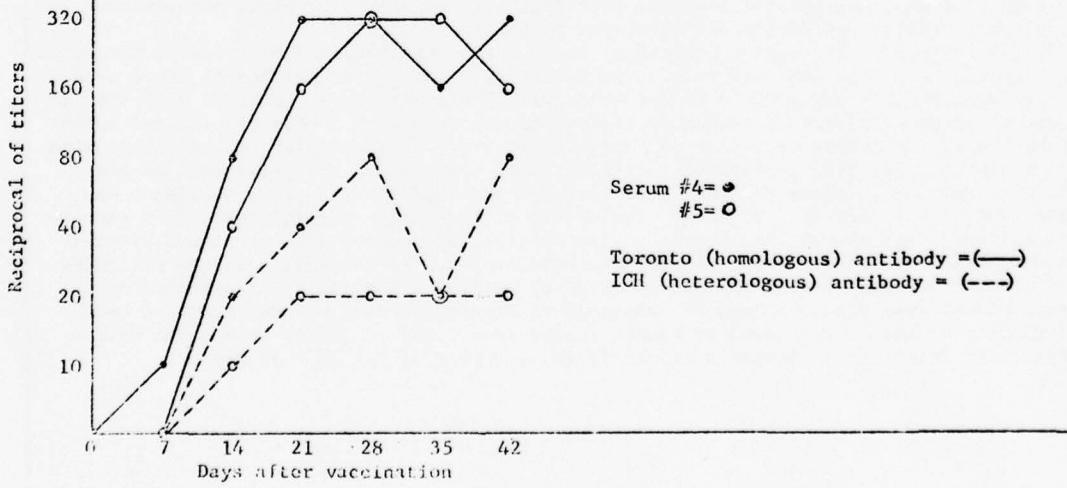


Test performed with primary dog kidney cells.

**Fig 5a** Homologous and heterologous serum antibody titers in dogs detected by enzyme-linked immunosorbant assay by time after vaccination with Toronto virus.



**Fig 5b** Homologous and heterologous serum antibody titers in dogs detected by neutralization test by time after vaccination with Toronto virus.



1. Test performed with primary dog kidney cells.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>b</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL
3. DATE PREV SUMRY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCYT <sup>b</sup> U	6. WORK SECURITY <sup>b</sup> U	DA OB 6444	77 10 01	DD-DR&E(AR)636
7. REGARDING <sup>b</sup>				8. DISPN INSTRN <sup>b</sup> NA NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT
10. NO./CODES: a. PRIMARY b. CONTRIBUTING c. CONTRIBUTING	PROGRAM ELEMENT 62770A	PROJECT NUMBER 3M762770A802	TASK AREA NUMBER 00	WORK UNIT NUMBER 012		
11. TITLE (Precede with Security Classification Code) (U) Diseases of the Military Dogs						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>b</sup> 010100 Microbiology 005900 Environmental Biology						
13. START DATE 68 07	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House		
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE PRECEDING FISCAL YEAR 77	19. PROFESSIONAL MAN YRS CURRENT 3	20. FUNDS (in thousands) 276
a. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. AMOUNT: e. KIND OF AWARD:	f. CUM. AMT.	78	2.5	300		
19. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research Washington, DC 20012 ADDRESS:				20. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Division of Veterinary Resources ADDRESS: Washington, DC 20012 PRINCIPAL INVESTIGATOR (Punish SSAN if U.S. Academic Institution) NAME: Stephenson, Edward H., LTC, DVM TELEPHONE: 202 427-5378 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]		
21. GENERAL USE Foreign Intelligence not considered				ASSOCIATE INVESTIGATORS NAME: Keefe, Thomas J., LTC, VC NAME: Binn, L.N., Ph.D.		
22. KEY WORDS (Precede EACH with Security Classification Code) (U) Military Dog; (U) Ehrlichia canis; (U) Canine; (U) Cell Hybridization; (U) Peritoneal macrophage; (U) Parainfluenza SV-5						
23. TECHNICAL OBJECTIVE. <sup>b</sup> 24. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) To investigate diseases and/or conditions affecting or associated with the military dog to enhance diagnosis, treatment and control. 24. (U) Conventional epidemiological, pathological, and microbiological methods are employed; special procedures are developed as needed. 25. (U) 76 10 - 77 09 Canine peritoneal macrophages were obtained by repeated peritoneal lavage of dogs at 14 day intervals. The macrophages and canine peripheral blood monocytes were equally susceptible to infection with Ehrlichia canis. Somatic cell hybrids were obtained by fusion of canine peritoneal macrophages with SV-40-transformed human fibroblasts. A hybrid cell line was established that was susceptible to infection with E. canis that had been propagated in canine blood monocytes. The prevalence of ehrlichial antibodies among military dogs from USA and USAF installations worldwide was greatest in dog sera from SE Asia. Evaluation of the canine parainfluenza SV-5 vaccine in military dogs showed that intramuscular vaccination induced more rapid and significantly higher SN titers than subcutaneous vaccination. Serological response following IM vaccination was similar to that produced by natural infection. An epizootic of gastrointestinal disease among US Army dogs in Miesan, Germany was demonstrated serologically to have been caused by canine coronavirus. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.						

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M762770A802 MILITARY ANIMAL RESOURCES DEVELOPMENT

Task 00 Military Animal Resources Development

Work Unit 012 Diseases of the Military Dog

Investigators:

Principal: LTC E. H. Stephenson, VC

Associates: L. N. Binn, PhD; LTC T. J. Keefe, VC; R. L. Marchwicki, BS;  
I. E. Hemelt, AB; SP4 E. Vinson; SP4 A. Robinson; PFC B.  
Ermeling; PFC R. von der Porten; and PFC L. Palmer

Description:

To define, study, diagnose, and control known and potential infectious diseases of military dogs. A major effort is directed toward developing an alternate *in vitro* method for cultivating Ehrlichia canis in lieu of the primary canine peripheral blood monocyte. Additional studies concern the epidemiology, diagnosis, treatment, and control of other disease agents or conditions affecting the military dog.

During the reporting period, research activities have included: (1) attempts at cultivation of E canis in cells other than primary canine peripheral blood monocytes, (2) evaluation of parainfluenza SV5 vaccine for the prevention of respiratory disease in recruit dogs, and (3) virus studies of an outbreak of gastrointestinal disease in US military dogs in Germany.

1. Canine peritoneal macrophages: Cultivation and infection with Ehrlichia canis.

Canine peritoneal macrophages were obtained by repeated peritoneal lavage of dogs at 14 day intervals. Intraperitoneal administration of sterile mineral oil increased the leukocyte yield approximately 20-fold and the macrophage recovery approximately 35-fold. Cell recovery was maximum 7 to 21 days after administration of oil and declined slightly by 35 days. Restimulation with a 2nd injection of oil promptly revitalized cell recovery. Peritoneal macrophage cultures were well established by 6 days after seeding and were maintained for at least 30 days. Initially, cultured macrophages were mononuclear, but binuclear cells appeared after 6 days' cultivation and multinucleated giant cells were observed after 14 days. Canine peritoneal macrophages and peripheral blood monocytes were equally susceptible to infection with Ehrlichia canis. Infected cells were detected by 60 hours after inoculation, and replication

was evident by 12 to 18 days. A detailed report has been accepted for publication.

2. Somatic cell hybrids of canine peritoneal macrophages and SV40-transformed human cells: Derivation, cultivation, and infection with *Ehrlichia canis*.

Canine ehrlichiosis continues to be a significant disease problem among military dogs, especially those deployed in tropical and subtropical areas.<sup>2</sup> Further, the prevalence of the disease in the US is greater than the sporadic reported cases indicated, as evidenced by the epizootic that occurred around Phoenix, AZ, in 1975.<sup>3</sup>

Significant advancements have been made relative to *ehrlichiae* and the disease they cause. However, continued productive research in this area requires propagation of the agent in large numbers and development of a reproducible method for quantification. Although *E. canis* readily replicates in primary cultures of canine peripheral blood monocytes<sup>4</sup> and peritoneal macrophages,<sup>1</sup> neither of the criteria can be satisfied. Investigative efforts, therefore, have and are being aimed at the development of an alternate in vitro cultivation system. Initially, attempts were made to infect approximately 12 different cell lines of types using direct inoculation, inoculation following irradiation of the cells, and inoculation after treatment of the cells with DEAE-dextran.<sup>5</sup> Since none of the cells tested could be infected with *E. canis*, emphasis was changed to the development of a somatic hybrid cell that would support the growth of the organisms. The purposes of this study were to: (1) obtain a somatic hybrid cell between canine peritoneal macrophages and SV40-transformed human cells, (2) characterize the somatic hybrid cells, and (3) evaluate the susceptibility of the somatic hybrid cells to infection with *E. canis*.

Cells. Canine peritoneal macrophages were collected and cultured according to described procedures.<sup>1</sup> SV40-transformed human skin fibroblasts (LNSV), which are deficient in Hypoxanthine Guanine Phosphoribosil Transferase (HGPRT), were obtained from Dr. C. M. Croce, Wistar Institute of Anatomy and Biology, Philadelphia, PA.

Cell hybridization. Canine peritoneal macrophages were fused with LNSV cells in the presence of UV-inactivated Sendai virus according to the method described by Croce et al.<sup>6</sup> The fused cultures were selected in hypoxanthine-aminopterin-thymidine (HAT) medium.<sup>7</sup> Hybrid cell colonies, growing in HAT selective medium, were picked and propagated in HAT medium.

Growth characteristics. Studies on the growth rate of the hybrid cells were made by seeding  $2.5 \times 10^6$  cells into each of 21- 75 cm<sup>2</sup> plastic culture flasks that contained 30 ml of HAT medium supplemented with 10% fetal calf serum (FCS). Cultures were incubated at 37 C. At 24 hour intervals, the cells in each of 3 culture flasks were dispersed with trypsin-EDTA and counted in triplicate using trypan blue exclusion to indicate cell viability.

Karyotypic analysis. Giemsa (G-) banding staining of the metaphase chromosomes of parental and hybrid cells was performed by a modification of the method described by Seabright.<sup>8,9</sup>

Enzyme analysis. Vertical starch gel electrophoresis was performed as described by Nichols and Ruddle.<sup>10</sup> Tissue culture cells used for enzyme extraction were somatic hybrid, LNSV, canine peritoneum, and canine kidney cells. The enzymes were extracted by homogenization or multiple freeze-thaw cycles in 1:20 Tris-EDTA-borate buffer, pH 8.6, at concentrations of  $8 \times 10^7$  cells/ml. The samples were then centrifuged at 40,000 x g for 60 minutes. Staining of the gels for specific enzymes was according to described methods.<sup>10,11</sup> Enzymes analyzed included galactose-6-phosphate dehydrogenase (Gal6PD), glucose-6-phosphate dehydrogenase (G6PD), glucose phosphate isomerase (GPI), glutamate dehydrogenase (GD), lactic dehydrogenase (LDH), mannose phosphate isomerase (MPI), nucleoside phosphorylase (NP), and phosphoglucomutase (PGM).

Infection of somatic hybrid cells with E canis. Leighton culture tubes with 9 x 35 mm glass coverslips and 25 cm<sup>2</sup> plastic culture flasks were seeded with a suspension of cells at a density that would yield a 60 to 70% monolayer after 48 hours incubation at 37 C. Culture medium was Eagle's minimal essential medium in Earles' balanced salt solution (EMEM) supplemented with 1 mM glutamine, 10 mM each of nonessential amino acids (NEAA), and 10% FCS. Inoculum was comprised of E canis that had been propagated in primary canine peripheral blood monocytes. When greater than 90% of the detached cells in infected monocyte cultures contained 1 or more morulae of E canis, the tissue culture medium was collected and centrifuged at 2,000 x g for 30 minutes at 4 C. The resulting pellet was resuspended in EMEM containing glutamine and NEAA to yield the inoculum. Growth medium was removed from the hybrid cell cultures. A 2.0 ml aliquot of inoculum was transferred to each 25 cm<sup>2</sup> flask culture and 0.4 ml to each Leighton tube culture. After a 2 to 3 hour adsorption period, the excess inoculum was removed and the cultures were fed with maintenance medium, which consisted of EMEM that contained glutamine, NEAA, 5% FCS, and 0.5 ug of cycloheximide/ml. Inoculated cultures were maintained at 37C. Maintenance medium was changed 24 hours after

inoculation and every 3 to 4 days thereafter. Infection of the cells with E canis was monitored by staining Leighton tube coverslip cultures using the direct fluorescent antibody technique at selected time intervals after inoculation.

A total of 158 clones of hybrid cells was obtained from the fusion of canine peritoneal macrophages with LNSV cells. Of these, 10 were successfully cultured after individual isolation. One of the somatic hybrid cell lines (WRH-2) was selected for detailed study.

The WRH-2 cells were susceptible to infection with E canis that had been propagated in canine peripheral blood monocyte cultures. Infected cells were observed 5 days after infection, using the direct fluorescent antibody technique, and contained 1 or more small inclusions or morulae. After 10 days, the number of infected cells had increased with a concomitant increase in the size and number of morulae.

Since its original isolation, the WRH-2 cell line has been serially passaged 45 times. Monolayer subcultures grew to confluence in 4 to 6 days after a 3:1 split. The growth curve of WRH-2 cells in monolayer culture showed a logarithmic increase in the number of cells between 24 and 144 hours after seeding. The population doubling time was estimated to be 23 hours, and the cells grew to a maximum of  $2.0 \times 10^5$  cells/cm<sup>2</sup>.

Karyotypic analysis showed a selective segregation of the canine chromosomes. Definition of the specific canine chromosomes in the WRH-2 cells, however, has not been completed. Using starch gel electrophoresis, the enzymes Gal6PD, G6PD, and LDH from WRH-2 cells had electrophoretic mobilities intermediate to the corresponding enzymes from parental cells. The migration patterns of other enzymes obtained from WRH-2 cells were identical to those of LNSV cells.

Heretofore, the only cells that had been shown to be susceptible to *in vitro* infection with E canis were primary canine peripheral blood monocytes<sup>4</sup> and peritoneal macrophages.<sup>1</sup> The ability to propagate E canis in a continuous cell line provides a means of obtaining large numbers of agents, which are needed for continued productive ehrlichial research. Successful subpassage of ehrlichiae propagated in WRH-2 cells will permit establishment of a quantification procedure, enhance the probability of development of an experimental immunogen, and allow detailed studies of the agent.

The hybrid cells, WRH-2, resulted from the fusion of canine peritoneal macrophages with human fibroblasts, which were deficient in HGPRT. Karyotypically there was a segregation of the canine chromosomes with a full complement of human chromosomes being maintained. The presence of such a chromosome content and the existence of a hybrid were also demonstrated by the results of enzyme electrophoretic mobilities. Additional characteristics of the hybrid cells are being examined, to include phagocytosis capabilities, surface antigens, ultrastructural characteristics, and immunoglobulin receptor sites.

A cell culture system, other than primary canine monocytes, was sought for propagation of E canis. Somatic cell hybrids were obtained by fusion of canine peritoneal macrophages with SV40-transformed human fibroblasts (LNSV). A cell line (WRH-2) was established from a single isolated hybrid clone. The WRH-2 cells were susceptible to infection with E canis that had been propagated in canine peripheral blood monocytes. Infected cells observed 5 days after infection, using the direct fluorescent antibody technique, contained one or more small inclusions or morulae. After 10 days, the number of infected cells had increased with a concomitant increase in the size and number of morulae. The WRH-2 cell line has been serially passaged 45 times and has a population doubling time of approximately 23 hours. Karyotypic analysis showed a selective segregation of canine chromosomes. Starch gel electrophoresis was used for enzyme characterization. Galactose-6-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, and lactic dehydrogenase from WRH-2 cells had electrophoretic mobilities intermediate to the corresponding enzymes from parental cells. The migration patterns of other enzymes obtained from WRH-2 cells were identical to those of LNSV cells.

### 3. Evaluation of parainfluenza SV5 vaccine for the prevention of respiratory disease in recruit military dogs.

For more than 10 years, epizootics of respiratory disease have seriously disrupted the training and deployment of military dogs at the procurement and training centers. During these epizootics nearly 25% of the dogs developed signs of respiratory disease and laboratory studies have consistently incriminated SV5 as the primary etiologic agent.<sup>12,13</sup> Nearly all the remaining susceptible dogs had inapparent infections. In December 1974, a multivalent vaccine containing attenuated SV5 was licensed by the USDA for the immunization of dogs.<sup>14</sup> The manufacturer indicated that the vaccine could be given by either subcutaneous (SC) or intramuscular (IM) routes but, the IM route was slightly more antigenic. In May 1975, a collaborative study was begun to evaluate the vaccine in recruited military dogs at Lackland Air Force Base (LAFB), Texas. The vaccine was given initially by the SC route as was customary for the older components of

the vaccine, i.e., canine distemper (CD), infectious canine hepatitis (ICH) and leptospira (L). The vaccine caused no untoward reactions and the SV5 component was not communicable. However, the response was modest, as unacceptably low titers of serum neutralizing antibody developed in 90% of the dogs (WRAIR Annual Report 1975-76). Further studies were conducted to ascertain whether or not the IM route of administration would be significantly more antigenic. This report summarizes the results of that study.

The study involved approximately 50 dogs, which were divided into 4 groups. Dogs in 2 groups were inoculated twice with the new SV5-CD-ICH-L vaccine on days 0 and 30; one group by the IM route and the other by the SC route. The dogs in remaining 2 groups were inoculated once with CD-ICH-L vaccine, one group by each route. The dogs were bled on arrival and before vaccination at LAFB and at intervals thereafter as indicated below. Serums were sent to the WRAIR for SV5 neutralization tests. The vaccinated dogs were examined for signs of disease and reaction to vaccination.

Signs of disease or reactions to vaccination were not observed. Serological tests did not detect any inapparent SV5 infections or spread of the vaccine virus to dogs given the CD-ICH-L vaccine. The findings indicated that both the IM and SC routes were safe for the immunization of dogs.

The antibody response of seronegative dogs given SV5 vaccine by the IM and SC routes and evaluated with the 3X84 strain are summarized in Tables 1 and 2. Following SC vaccination the response was comparable to that described in the previous Annual Report (WRAIR Annual Report 1975-76). Fifteen days after immunization, low levels of neutralizing antibody were detected in approximately half the dogs. Following the second dose, nearly 90% of the dogs were seropositive with a geometric mean titer (GMT) of 18.6 (Table 1). In contrast, most dogs vaccinated by the IM route had detectable antibody 7 days after the first dose, and at 14 and 28 days the GMT were greater than that of the dogs given 2 SC doses. Following the IM booster dose, an approximate 10-fold increase in GMT to 300.6 was achieved. In general, the level of antibody produced 1 month after the second dose was maintained through 6 months. The findings clearly indicated that vaccination by the IM route produced significantly faster and higher antibody responses.

Additional serum neutralization tests on these post vaccination serums with 2 other strains of canine SV5 gave similar results (Table 2). Serums from dogs vaccinated IM were neutralized earlier and to much higher titer than were serums from SC vaccinated dogs. Moreover, the levels of antibody observed after 2 IM doses were comparable to those obtained 21 days after field infection of military dogs (Table 3).

In summary, the attenuated parainfluenza SV5 vaccine was safe and non-communicable in military dogs. After 2 IM doses of vaccine, the levels of antibody were equivalent to those produced by natural infection. High levels of antibody persist for at least 6-months. The findings clearly indicated that the IM route of immunization produced significantly higher and more persistent titers of neutralizing antibody than those evoked by SC vaccination. Indeed, the same vaccine when used in the field to prevent infection or stop an epizootic may be quite effective when administered IM, but appear to be questionable or of little value if given SC. Based on these vaccine trials, an initial regimen of two IM vaccinations, 30 days apart, followed by an annual booster is recommended.

4. Virus studies of an outbreak of gastrointestinal disease in US Military Dogs in Germany.

During February 1977 an epizootic of gastrointestinal disease occurred in 24 US military working dogs (MWD) at the Miesau Army Depot in Germany. Most affected dogs had multiple episodes of vomiting over a 24-48 hour period and a few dogs had diarrhea. Laboratory tests in Germany were negative for parasites and for Salmonella sp. Because the etiology was unknown, a request for virus studies was forwarded to the Division of Veterinary Medicine, WRAIR. Subsequently, specimens of vomitus and feces for virus isolation tests, and acute and convalescent serum specimens for serological tests were sent and examined. Viruses were not recovered from the specimens in either primary dog kidney cells or the Walter Reed Canine Cell line. Failure to recover viruses may have reflected the limited number of specimens obtained from sick dogs at the end of the epizootic. However, 5 of 10 dogs affected showed significant increases in titers of serum neutralizing antibody to the canine coronavirus and the antigenically related transmissible gastroenteritis virus of swine. The findings clearly indicated that a coronavirus was present during the epizootic and infected a large number of sick dogs. It is of interest to note that a canine coronanavirus was isolated from a winter outbreak of gastrointestinal disease in US MWD in Germany during 1971<sup>15</sup> and also was recovered from German Shepherd puppies with diarrheal disease at Edgewood Arsenal, MD (WRAIR Annual Report 1974 to 1975). These new observations provide additional evidence that coronaviruses may be an important cause of epizootic diarrhea in dogs.

In summary, coronavirus infections were demonstrated in 50% of sick US military working dogs in Germany with gastrointestinal disease. The findings provide further evidence that coronavirus may be an important cause of epizootic gastrointestinal disease of dogs.

Project 3M762770A802 MILITARY ANIMAL RESOURCES DEVELOPMENT

Task 00 Military Animal Resources Development

Work Unit 012 Diseases of the Military Dog

Literature Cited.

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Table 1: Comparison of the parainfluenza SV5 neutralizing antibody response of seronegative dogs given attenuated CDV-ICH-SV5 vaccine by either the subcutaneous or intramuscular routes

Vaccination schedule	Day post vaccination examined	No. of dogs with antibody titer <sup>1</sup>						Geom. mean titer	No. sero positive
		0 <sup>2</sup>	2	4	8	16	32		
<u>subcutaneous route</u>									
1	0	7	12	2	4	2	2	1.0	2/14 (14)
	15	5	1	1	6	2		3.1	9/14 (62)
	30	6	1	1	2	1	4	2.8	8/14 (57)
2	30	45	2	1	2	2	1	18.6	12/14 (86)
	70	1	3	4	3	3		9.3	13/14 (93)
	180	2	1	1	1	1	2	5.7	6/8 (75)
<u>intramuscular route</u>									
1	0	7	2	3	1	3	2	1	5.0
	15	15	1	1	1	5	3	4	10/12 (83)
	30	30	1	1	1	4	5		13/13 (100)
2	30	45	1	1	1	1	1	1	33.8
	70	70	1	1	1	1	1		13/13 (100)
	180	180	1	1	2	2	2	1	300.6
									103.4
									64.0
									8/8 (100)

1. Test virus 3X84  
 2. Less than 1:2

Table 2: Comparison of the neutralizing antibody response to three canine parainfluenza SV5 isolates of military dogs given canine parainfluenza vaccine.

Vaccination schedule		Day post vaccination examined	Antibody response to SV5 strains				
Dose	Day (Route)		3X84 <sup>1</sup>		Tramp <sup>2</sup>		D008 <sup>3</sup>
		GMT	No. positive	GMT	No. positive	GMT	No. positive
			No. tested (%)		No. tested (%)		No. tested (%)
1 (SC) <sup>4</sup>	0	7	0	0/7	{ 0 }	0/7	{ 0 }
		15	1.8	2/7	{ 29 }	4/7	{ 57 }
		30	1.3	1/7	{ 14 }	2.2	{ 43 }
		45	11.9	5/7	{ 71 }	5/7	{ 71 }
		70	8.0	5/6	{ 83 }	14.3	{ 83 }
1 (IM) <sup>5</sup>	0	7	5.0	10/12	{ 83 }	16.0	11/12 { 92 }
		15	25.9	13/13	{ 100 }	57.5	13/13 { 100 }
		30	33.8	13/13	{ 100 }	57.5	13/13 { 100 }
		45	300.6	13/13	{ 100 }	600.7	13/13 { 100 }
		70	103.4	13/13	{ 100 }	256.0	13/13 { 100 }

1. Ft Benning 1966 isolate
2. Lackland Air Force Base, Texas 1973 isolate
3. WRAIR Laboratory dog 1964 isolate and parental virus for alternative vaccine
4. SC = subcutaneous
5. IM = intramuscular

Table 3: Neutralizing antibody titers to three canine SV5 strains in military dogs following respiratory infections at Lackland AFB, 1973 - 1974.

Challenge virus (origin)	No. dogs examined	No. dogs with titer <sup>1</sup>				Geom. mean titer
		64	128	256	512	
3X84 (Ft Benning, 1966)	21	3	4	9	5	219
Tramp (Lackland AFB, 1973)	11	1	2	4	1	3
D008 <sup>2</sup> (WRAIR, 1964)	16		2	3	6	2
					3	535

1. Approximately 21 days post onset.  
 2. Recovered from a laboratory dog and parental virus of P-M SV5 vaccine.

Project 3M762770A803

MALARIA PROPHYLAXIS AND TREATMENT

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>9</sup>	2. DATE OF SUMMARY <sup>9</sup>	REPORT CONTROL SYMBOL
				DA DA 6520	77 09 30	DD-DR&E(AR)636
3. DATE PREV SUMRY 76 10 01	4. KIND OF SUMMARY K. Completion	5. SUMMARY SCY <sup>9</sup> U	6. WORK SECURITY <sup>9</sup> U	7. REGARDING <sup>9</sup> NA	8. DA DISGN INSTN <sup>9</sup> NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: <sup>9</sup> a. PRIMARY 62770A	PROGRAM ELEMENT	PROJECT NUMBER 3M762770A8 03		TASK AREA NUMBER 00	10. LEVEL OF SUM WORK UNIT NUMBER 081	
b. CONTRIBUTING	c. CONTRIBUTING CARDS 114F					
11. TITLE (Pecode with Security Classification Code) (U) Host Responses to Malaria						
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>9</sup> 002600 Biology						
13. START DATE 64 07	14. ESTIMATED COMPLETION DATE 77 09		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House		
17. CONTRACT/GRANT			18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS	20. FUNDS (In thousands)	
A. DATES/EFFECTIVE: NA	EXPIRATION:		FISCAL YEAR PRECEDING 76 CURRENT 77	4	197	
B. NUMBER: <sup>9</sup>	C. TYPE:		AMOUNT:	4	239	
D. KIND OF AWARD:	E. CUM. AMT.					
19. RESPONSIBLE DOD ORGANIZATION			20. PERFORMING ORGANIZATION			
NAME: <sup>9</sup> Walter Reed Army Institute of Research			NAME: <sup>9</sup> Walter Reed Army Institute of Research Div of CD&I			
ADDRESS: <sup>9</sup> Washington, D. C. 20012			ADDRESS: <sup>9</sup> Washington, D. C. 20012			
RESPONSIBLE INDIVIDUAL NAME: GARRISON RAPMUND, COL TELEPHONE: (202) 576-3551				PRINCIPAL INVESTIGATOR (Punish <del>NAME</del> if U.S. Academic Institution) NAME: <sup>9</sup> BURKE, COL J. C. TELEPHONE: (202) 576-2273 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]		
21. GENERAL USE Foreign intelligence not considered				ASSOCIATE INVESTIGATORS NAME: MOON, A. P. DA NAME: [REDACTED]		
22. KEYWORD (Pecode EACH with Security Classification Code) (U) Malaria; (U) Irradiation; (U) Cellular Immunity; (U) Lymphocyte; (U) Tolerance; (U) Serology; (U) Cyclophosphamide						
23. TECHNICAL OBJECTIVE, <sup>9</sup> 24. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number. Pecode rest of each with Security Classification Code) 23. (U) To study the physiopathology, immunology and serology of malaria--a disease of prime concern to troops stationed in tropical areas, causing considerable loss of man-days due to severe morbidity and mortality. 23. (U) Evaluation of cellular and humoral immunity in rodent malaria by passive-transfer protection studies with lymphocytes, macrophages and serum. Study of mechanisms of immune response to antigens of rodent malaria through induction of antigen-specific unresponsiveness (tolerance) to <i>P. berghei</i> . 25. (U) 76 10-77 09 Both nonspecific and specific chemosuppression of the immune response to protective antigens of <i>Plasmodium berghei</i> in mice were investigated. Gamma-irradiated infected mouse erythrocytes, sheep erythrocytes, and cyclophosphamide were used. Animals treated with malarial antigen and cyclophosphamide developed specific immunosuppression. There is a delicate balance between immunological responsiveness and unresponsiveness which suggests that suppressive phenomena may coexist with partial immunity. Studies under this work unit will be continued under work unit 083. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.						

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Project 3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT

Work Unit 081 Host Responses to Malaria

Investigators.

Principal: COL J. C. Burke, MSC

Associate: W. L. Bowie, C. L. Diggs, K. M. Esser, W. H. Hildreth,  
S. M. Phillips, R. A. Wells

1. Cyclophosphamide-induced tolerance to the protective immunogens of rodent malaria.

Man is exceedingly susceptible to malaria during the first five years of life and only acquires a significant clinical immunity after years of exposure to the infection. Even after a degree of immunity is acquired, a long and varied clinical course, characterized by a number of exacerbations and remissions of the disease process is observed. This clinical picture is compatible with the hypothesis that a form of functional tolerance may play at least a partial role in influencing the outcome of the disease.

Although there is currently no direct evidence to suggest that functional tolerance is operative in malaria, there is some evidence for the existence of this phenomenon in other infectious systems. In addition, extensive studies have been performed in the area of specific drug induced specific immunological hyporeactivity to several different potentially antigenic materials.

A greater understanding of basic host defense mechanisms in malaria is likely to aid in the development of effective immunoprophylactic measures. In particular, if tolerance, regardless of mechanism, is operative, the recognition and ultimate manipulation of this phenomenon would be of great importance. The present report summarizes studies designed to explore the induction of immunological tolerance to the protective immunogen of the rodent malaria parasite Plasmodium berghei through the combined administration of immunogen and cyclophosphamide.

Animals.--Outbred male Walter Reed (Wrm-ICR-BR) mice 10 to 11 weeks of age were used throughout this study.

Drug. Cyclophosphamide (CY, CYTOXAN) was obtained from Meade-Johnson and Company, Evansville, Indiana. The drug was freshly reconstituted before use and administered in 4 i.p. injections at 24 hour intervals at a dose level of 40 mg/kg/day. Unless otherwise stated, the cytoxan was injected in two series, commencing 48 hours after exposure to  $\gamma$ -Pb. The interval between the two series was one week.

Parasites.--The NYU-2 strain of the rodent malaria parasite Plasmodium berghei was utilized. Challenge with the blood forms of parasite resulted in lethal infections in 100% of nonimmunized mice.

Immunization techniques.--(2) Malaria organisms: Mice were immunized by the i.p. injection of  $5 \times 10^8$  irradiated parasitized erythrocytes ( $\gamma$ Pb) as previously described in detail. Briefly, the immunogen was derived from the blood mice infected 3-4 days previously with  $2 \times 10^7$  parasitized red blood cells. The blood was collected in chilled sodium citrate solution prior to gamma irradiation at  $2 \times 10^3$  rads from a  $^{60}\text{Co}$  source. The  $\gamma$ Pb were centrifuged at 900 g for 10 minutes at  $4^\circ\text{C}$  and the supernatant fluid withdrawn. The cells were resuspended to the original volume in phosphate buffered saline (PBS), 0.15M, pH 7.3. The number of infected cells were determined by counting in a hemocytometer, utilizing Nile blue stain. (b) Sheep red blood cells: Mice were immunized with  $5 \times 10^8$  washed SRBC injected i.p.

Evaluation of protection against malaria.--The mice were challenged with  $2 \times 10^4$  blood forms of the parasite and their susceptibility assayed by mortality and parasitemia. The daily cumulative mortality of each group was recorded at 24 hour intervals for 30 days after challenge. Protection was also evaluated by counting the percentage of infected cells among a total of 300 erythrocytes in thin blood films made on days 6, 9 and 12 after challenge.

Determination of response to SRBC.--(a) Hemagglutination: Sera were processed 5 days after immunization and were frozen at  $-80^\circ\text{C}$  until testing for antibody activity. Sera were thawed at  $37^\circ$  and heat inactivated at  $56^\circ\text{C}$  for 60 minutes. Doubling serial dilutions of the sera were produced with chilled PBS and placed in plastic microtiter plates. Hemagglutination was evaluated after overnight incubation utilizing a final SRBC concentration of 0.75%. Equivocal reactions were considered negative. The final titer was recorded as the reciprocal log<sub>2</sub> dilution at which a positive agglutination reaction was noted.

(b). Hemolysin: The SRBC antigen and sera were suspended in 0.03M sodium buffered diethylbarbiturate 0.17M NaCl, pH 7.4 containing 0.1% gelatin. The SRBC antigen was standardized spectrophotometrically so that a 1:23 dilution of hydrolysate yielded an optical density of 0.95-1.05 at a wave length of 512nm. Controls included a rabbit amboceptor and a reagent control of pooled mouse anti-SRBC. Magnesium and calcium chloride were added to the buffer at final concentrations of  $1 \times 10^{-3}$  M  $\text{Mg}^{++}$  and  $1.5 \times 10^{-4}$  M  $\text{Ca}^{++}$ . Previously reconstituted guinea pig complement was thawed from  $-80^\circ\text{C}$  and diluted 1:40 in chilled veronal buffer immediately before use. The optical density of each tube was determined and expressed as a percentage of that obtained from the completely lysed controls. The amount of serum required for 50% lysis ( $\text{HD}_{50}$ ) was estimated by interpolation on log serum dose-probit hemolysis plots.

Experimental design.--Unless otherwise specified, experiments evaluating drug induced modulation of experimental immunization consisted of the following experimental groups:

Animals pretreated with  $\gamma$ Pb and CY. Mice were pretreated with two doses of  $5 \times 10^8$   $\gamma$ Pb and two series of CY with administration of the drug beginning 48 hours after the  $\gamma$ Pb. The interval between these two pretreatment doses of  $\gamma$ Pb was 1 week.

CY pretreatment control group. These animals were pretreated as described above (1) except that initial pretreatment did not include the concomitant exposure to  $\gamma$ Pb, but rather CY alone.

$\gamma$ Pb pretreatment control group. Animals were pretreated as described above except that they were not pretreated with CY but rather received  $\gamma$ Pb only.

Non-pretreated control group. These animals were given no pre-treatment.

Beginning two weeks after the second  $\gamma$ Pb pretreatment of groups 1 and 3, all four groups were given an immunization regimen which consisted of 2 i.p. injections of  $5 \times 10^8$   $\gamma$ Pb two weeks apart.

Susceptibility controls. These animals were neither pretreated nor immunized but served to prove infectivity of the inoculum at the time of challenge (one week after the last immunizing dose).

Statistical analysis.--Mortality rates were evaluated by the Mann-Whitney U test. Parasitemia values and SRBC hemagglutination titers were analyzed by T-test and the 95% confidence limits were established for each of the experimental groups.

Effect of pre-immunization with  $\gamma$ Pb upon subsequent resistance to challenge.

The relationship between preexposure to  $\gamma$ Pb and the induction of immunity was first determined. Figure 1 summarizes the cumulative mortalities of challenged mice, previously treated with 0, 1, 2 or 4 doses of  $\gamma$ Pb. Unexposed animals were completely susceptible showing 90% mortality on about day 11 after challenge and invariable (100%) mortality by day 30. Mice given a single dose of  $10^9$   $\gamma$ Pb showed only slight protection with 60% deaths by day 13 and cumulative mortality of 90% by day 30. Mice given 2 doses of  $5 \times 10^8$   $\gamma$ Pb showed moderate protection with cumulative mortalities of 7% through day 13 and 75% by day 30. Mice given 4 doses of  $\gamma$ Pb showed the greatest protection with no deaths occurring until the third week after challenge and with a cumulative mortality of approximately 25% by day 30. On the basis of these experiments a regimen of 2 doses of  $5 \times 10^8$   $\gamma$ Pb was selected since this resulted in a reproducible, moderate degree of immunity of use in the evaluation of subsequent studies on immunosuppression of protection against malaria.

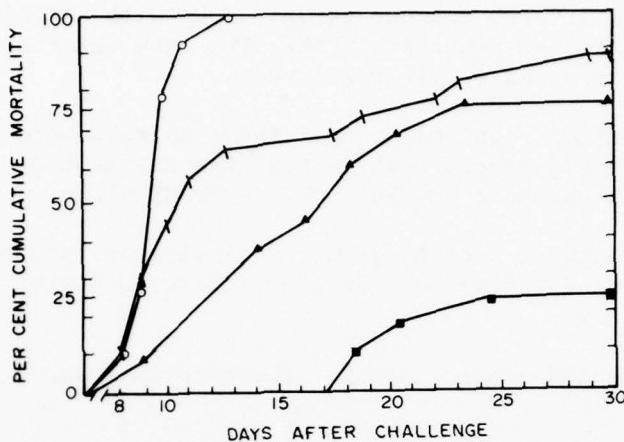


Figure 1. Effect of immunization with *P. berghei* parasitized erythro-( $\gamma$ Pb) on mortality after challenge with nonirradiated parasites. The respective initial treatments were: (○), no  $\gamma$ Pb; (▽),  $10^6$  Pb in 1 dose; (▲), 2 doses of;  $5 \times 10^6$   $\gamma$ Pb; (■), 4 doses of  $5 \times 10^6$   $\gamma$ Pb. Data points are composites of two experiments.

#### Effects of cyclophosphamide and $\gamma$ Pb upon subsequent resistance to challenge.

Following the establishment of a model for the measurement of immunity, the effects of CY and  $\gamma$ Pb pretreatment, both alone and in combination on subsequent immunization were compared. Previous studies have shown that the temporal relationship between the administration of the  $\gamma$ Pb and CY is critical for the demonstration of a synergistic effect on immunosuppression. Figure 2 illustrates the median survival times of groups of mice initially treated with 2 representative series of  $\gamma$ Pb and CY. The first administration of the drug started at the indicated time intervals before or after  $\gamma$ Pb. Following pretreatment, mice were given 2 doses of  $\gamma$ Pb alone and were later challenged with the parasite. The results indicated that the maximum difference between the experimental ( $\gamma$ Pb+CY) and drug control (CY) groups occurred when the drug series were begun 2 days after treatment with  $\gamma$ Pb. This regimen was thus selected for further investigations. Figure 3 illustrates the pooled mortality values of mice in 3 experiments, utilizing the regimen indicated. The interval between the last pretreatment ( $\gamma$ Pb+CY) and the first immunization dose was either 1 or 2 weeks. The rates and extents of mortality were similar in all experiments and exhibited the same qualitative relationships among experimental groups. Animals treated with  $\gamma$ Pb+CY prior to immunization showed mortalities which closely resembled those of nonpretreated, nonimmunized normal mice. There was 1 surviving animal among the 34 mice thus treated. The mortalities among the drug control animals were intermediate between the two groups

TABLE I

*Sheep erythrocyte hemagglutination titers of mice pretreated with selected regimens and assayed at specified time intervals*

Pretreatment	Immunization	Time of SRBC Challenge	No. of Ani- mals	Mean <sup>a</sup> Log <sub>2</sub> Recipro- cal Titer ± S.E. <sup>b</sup>	95% Confidence Limits
		1 week after pretreatment	10	6.4 ± 0.2	6.0-6.8
	$\gamma$ Pb(×2)		10	5.4 ± 0.3	4.8-6.0
	CY(×2)		10	5.0 ± 0.4	4.1-5.9
		2 weeks after pretreatment	10	10.1 ± 0.3	9.5-10.7
	$\gamma$ Pb + CY(×2)		23 <sup>c</sup>	6.8 ± 0.3	6.4-7.2
	CY(×2)		26	6.9 ± 0.2	6.5-7.3
	SRBC + CY(×2)		5	2.6 ± 0.3	1.9-3.3
		4 weeks after pretreatment	5	6.2 ± 0.4	5.2-7.2
	$\gamma$ Pb(×2)		6	6.2 ± 0.3	5.4-7.0
	$\gamma$ Pb(×2)	1, 2, or 4 weeks after pretreatment of the experimental mice	44 <sup>d</sup>	7.5 ± 0.1	7.3-7.7

<sup>a</sup> All groups significantly different (*t*-test, 95% confidence limits) from normal, SRBC-challenged controls.

<sup>b</sup> ± Mean standard error.

<sup>c</sup> One nonresponding animal excluded (titer <1:2).

<sup>d</sup> Three nonresponding animals excluded (titer <1:2).

TABLE II  
*Hemolysin titers of mice challenged with SRBC after treatment with  
selected regimens*

Treatment before SRBC	No. of Ani- mals	Mean Log <sub>2</sub> Reciprocal HD <sub>50</sub> Titer ± S.E. <sup>a</sup>	95% Confidence Limits
CY(×2)	6 <sup>b</sup>	3.8 ± 0.1	3.4-4.0
$\gamma$ Pb(×2)	6	3.6 ± 0.1	3.4-3.8
$\gamma$ Pb + CY(×2)	6	3.4 ± 0.1	3.0-3.8
	18	3.6 ± 0.1	3.3-3.9

<sup>a</sup> ± Mean standard error.

<sup>b</sup> One nonresponding animal excluded (titer < 1 HD<sub>50</sub> unit).

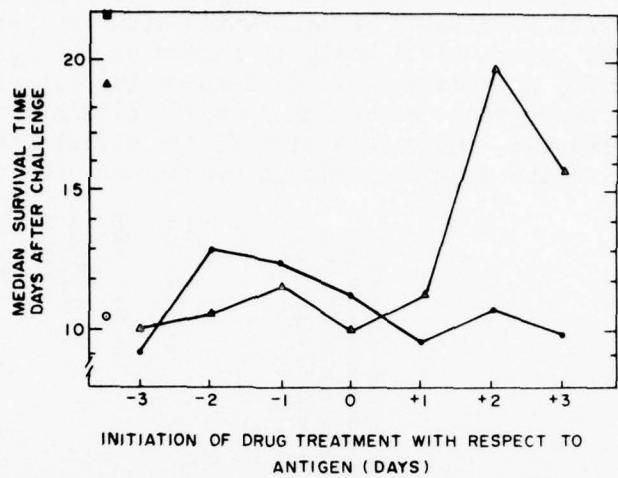


Figure 2. Median survival time of mice given selected initial treatments before immunization with  $\gamma$ Pb and challenge with *P. berghei*. The respective initial treatments were: (○), no treatment / challenge controls; (●),  $\gamma$ Pb + CY in two series; (△), CY in two series; (▲), no initial treatment/immune controls; (■),  $\gamma$ Pb in two series.

Since it was possible that cumulative mortality might be due to some factor other than the course of malaria *per se*, the effect of  $\gamma$ Pb+CY upon parasitemia was also evaluated. Figure 4 shows the mean parasitemia on selected days after challenge of animals in the experiments indicated in Figure 3. The mean parasitemia was highest in non-immunized mice and next highest in those pretreated with  $\gamma$ Pb+CY. The parasitemias of the CY pretreated and nonpretreated control groups closely resembled each other with a trend toward higher values in CY controls. The  $\gamma$ Pb pretreated animals exhibited the lowest parasitemia. Thus the effects of  $\gamma$ Pb+CY upon parasitemias in the various experimental groups closely paralleled the effects observed for cumulative mortalities.

#### Specificity of immunosuppression

The specificity of the observed suppression of the protective response to malaria was concurrently evaluated by testing the ability of subgroups of the treated mice to produce antibody to another antigen, SRBC. Table I illustrates the hemagglutination titers of mice initially exposed to selected antigen and drug regimens. All groups produced measurable antibody; although, as predicted, there was a marked reduction of titers where the initial treatment consisted of the homologous antigen (SRBC) + CY. Regardless of the pretreatment regimen animals in all groups were able to respond to the SRBC at the same time that their group counter-

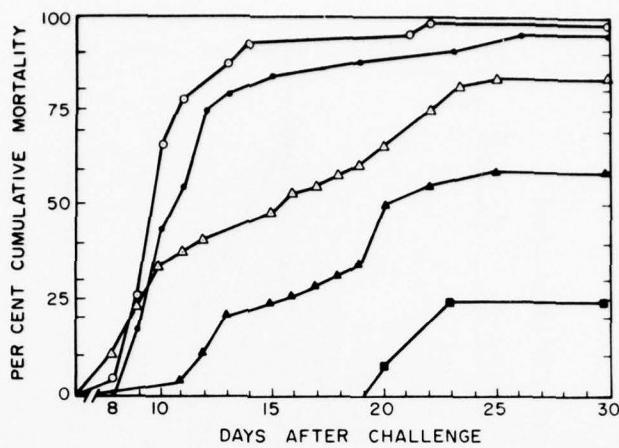


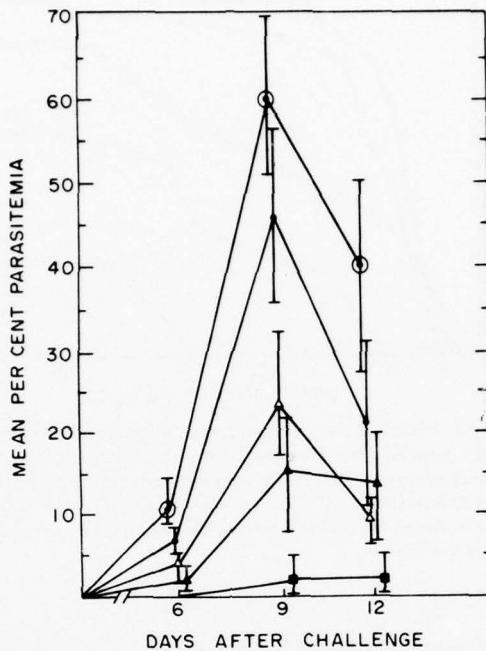
Figure 3. Post-challenge mortalities of mice after treatment with selected regimens. The respective initial treatments before immunization with  $\gamma$ Pb were: (○), no treatment/challenge controls; (●),  $\gamma$ Pb + CY in two series; (▲), CY in two series; (△), no initial treatment/immune controls; (■),  $\gamma$ Pb in two series. Data points are composites of three experiments.

parts were being immunized with  $\gamma$ Pb. Mice pretreated with CY showed a relatively small, but statistically significant, suppression of the anti-SRBC response when compared with non-drug treated, SRBC challenged mice. There was a smaller degree of suppression induced by  $\gamma$ Pb pre-treatment alone where this regimen preceded SRBC challenge by 1 week. Initial treatment with  $\gamma$ Pb+CY resulted in titers indistinguishable from animals receiving CY alone. It is noteworthy that where 2 doses of  $\gamma$ Pb alone preceded SRBC challenge by 2 weeks there was a marked enhancement of the formation of hemagglutinating antibody over that observed in the normal SRBC challenged groups.

Responsiveness to SRBC was also tested by hemolysin assays. In these experiments SRBC were given at the time of malaria challenge. The regimen employed was a 2 week interval in pretreatment with an additional 2 weeks between  $\gamma$ Pb doses in immunization followed by 1 week until challenge. Table II summarizes the titers of these groups of mice. Although the treatment regimen was associated with an identical modification (*op. cit.*) of the anti-malarial response, no effect upon the anti-SRBC response was noted.

#### Effect of additional antigen

Animals rendered hyporesponsive to  $\gamma$ Pb were given one additional dose of  $\gamma$ Pb on day 21 during the immunization phase in addition to the standard regimens of  $\gamma$ Pb on days 14 and 28. Figure 5 illustrates the effect on protective immunity on groups of mice treated in this way. In

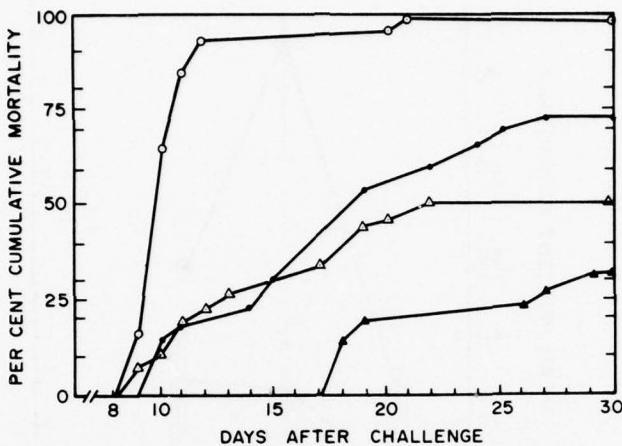


**Figure 4.** Parasitemias in mice challenged with malaria after treatment with selected regimens. The respective initial treatments before immunization with  $\gamma$ Pb were: (○), no treatment/challenge controls; (●),  $\gamma$ Pb + CY in two series; ( $\triangle$ ), CY in two series; ( $\blacktriangle$ ), no initial treatment/immune controls; (■),  $\gamma$ Pb in two series. The values of the latter group were from a single experiment while those in the other groups are composites of three experiments. Vertical bars represent the 95% confidence limits of respective mean data points.

contrast to the 2 dose  $\gamma$ Pb regimen (Figure 3) animals given 3 doses of  $\gamma$ Pb after  $\gamma$ Pb+CY exhibited immunity comparable to mice immunized with 2 doses of  $\gamma$ Pb alone prior to challenge and showed a death rate not unlike the CY control groups until the later stages of the 30-day test period. Nonimmunized and nonpretreated immunized controls displayed the greatest and least mortality respectively as was anticipated.

Figure 6 illustrates the reduction (as compared with Figure 4) in parasitemia of these same groups of mice with the administration of additional  $\gamma$ Pb. The parasitemia values again paralleled the mortality assays in indicating the extent of immunity.

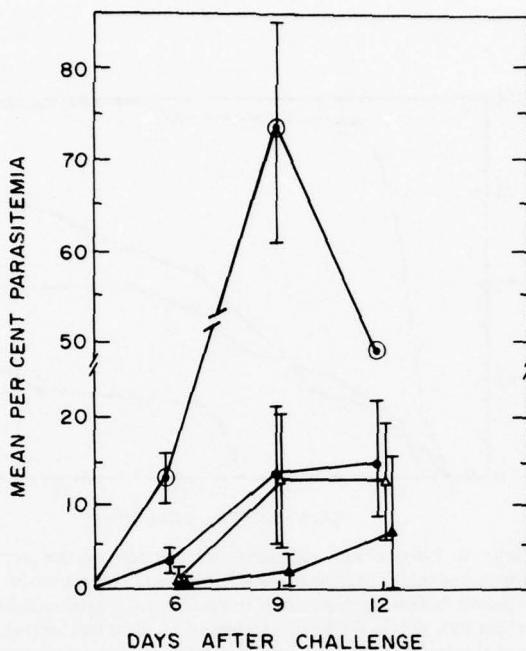
These studies demonstrate that a marked suppression of the immune response to murine malaria immunization results when malarial antigen is given concomitantly with cyclophosphamide.



*Figure 5.* Effect of one additional antigen dose on the mortalities of pretreated mice challenged with *P. berghei*. The respective initial treatments before immunization were: (○), no treatment/challenge controls; (●),  $\gamma$ Pb + CY in two series; ( $\Delta$ ), CY in two series; ( $\blacktriangle$ ), no initial treatment/immune controls. Data points are composites of two experiments.

The continued ability of mice pretreated with  $\gamma$ Pb and CY to produce as much SRBC antibody as those initially exposed to CY alone demonstrates the specificity of the  $\gamma$ Pb + CY-dependent suppression of the immune response to  $\gamma$ Pb. These results imply that mice treated with a malaria antigen and drug develop a specific immunosuppression which resembles immunologic tolerance in many respects; i.e., there is a more marked immunosuppression in immunogen plus drug-treated animals than in drug-treated controls in combination with a continued responsiveness to a second antigen (SRBC). Thus, this model resembles others in which there is minimal nonspecific immunosuppression by cyclophosphamide with a demonstrable enhancement of immunosuppression through the combined administration of specific antigen in conjunction with drug.

The present model also resembles others in its requirement for a confined time relationship between administration of antigen and drug



**Figure 6.** Effect of one additional dose of antigen on the parasitemias of pretreated mice challenged with *P. berghei*. The respective initial treatments before immunization were: (○), no treatment/challenge controls; (●),  $\gamma$ Pb + CY in two series; ( $\triangle$ ), CY in two series; ( $\blacktriangle$ ), no initial treatment/immune controls. Data points are composites of two experiments. The day-12 value of the challenge control group represents one surviving animal.

for the induction of tolerance. Suppression of the protective response was most selective when Pb was given 48 hr before each series of CY. The ability to demonstrate antigen-specific suppression, as opposed to general chemosuppression, thus rests on the apparent ability of the immune response to escape the general suppressive effects of the drug, but not the antigen dependent effects when administration is delayed for 2 days after treatment with malaria antigen. These results depart from those obtained with SRBC tolerance models in that superimposed non-specific chemosuppression is not as prominent in the SRBC model. In both cases it can be speculated that the clone of immunocompetent cells responsible for the protective immune response to malaria or for anti-SRBC production appears first to be stimulated by antigen to proliferate and thus become more vulnerable to the cytotoxic effects of cyclophosphamide. Reversal of antigen-specific protective immunosuppression occurs

more readily than reversal of tolerance to SRBC antigens. Suppressed mice exhibit a clear-cut immune response to a single additional dose of antigen given between the standard immunization phase doses used for other experiments. In contrast, additional antigen doses administered to SRBC-tolerant mice serve to maintain the tolerant state.

The phenomena observed might be explained in a number of ways since multiple models of induction of antigen-specific unresponsiveness have been described involving both well defined and parasitic antigens. Clonal deletion, as described above, if operative, must be incomplete since additional immunogenic stimulation results in augmented resistance to infection. Thus, the partial destruction of clones of specifically committed cells would allow a reversal of the tolerant state. Alternatively, the tolerance state may be due to a reversible inactivation of immunocompetent cells rather than their absolute destruction. It is likewise possible that the antigen-drug combination may lead to an alteration in the relative dominance of various modulating cell populations, for example, suppressor cells. Our results might also be explained in terms of changes in effector function. For example, an increase in the production of antibody which interacts with the antigenic determinants which are the normal target of the immune response but which do not result in parasite inactivation could explain the observed phenomena. A role for such an enhancing antibody in schistosomiasis has been suggested. Effector cell subpopulations such as monocytes might also be less effectively activated in this system.

A number of these possibilities are susceptible to experimental attack. The elucidation of such regulatory mechanisms should enhance the possibilities of the improved control of malarial through immuno-prophylactic and immunotherapeutic means.

Project 3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT

Work Unit 081 Host Responses to Malaria

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
3. DATE PREV SURY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY# U	6. WORK SECURITY# U	DA OA 6514	77 10 01	DD-DR&E(AR)636
7. REGADING# NA	8. DSBN INSTN# NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT			
10. NO./CODES: a. PRIMARY 62770A	PROGRAM ELEMENT 3M762770A802	PROJECT NUMBER 00	TASK AREA NUMBER 013	WORK UNIT NUMBER		
b. CONTRIBUTING 62770A	3M762770A803					
c. CONTRIBUTING CARDS 114F						
11. TITLE (Proceed with Security Classification Code)* (U) Biological Studies of Insect Infection and Disease Transmission						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS* 002600 Biology						
13. START DATE 65 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House			
17. CONTRACT/GRANT a. DATES/EFFECTIVE: NA b. NUMBER: c. TYPE: e. KIND OF AWARD:		EXPIRATION: f. CUM. AMT.	18. RESOURCES ESTIMATE FISCAL YEAR 77 CURRENcy 78	19. PROFESSIONAL MAN YRS 4.3 4.3	20. FUNDS (in thousands) 212 260	
19. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research ADDRESS: Washington, DC 20012		20. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Div of CD&I ADDRESS: Washington, DC 20012				
RESPONSIBLE INDIVIDUAL NAME: Raptmund, COL G. TELEPHONE: 202-576-3551		PRINCIPAL INVESTIGATOR (Furnish Name if U.S. Academic Institution) NAME: Gould, Dr. D. J. TELEPHONE: 202-576-3719 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED] ASSOCIATE INVESTIGATORS NAME: Schneider, Dr. I. NAME: Gargan, CPT T.				
21. GENERAL USE Foreign intelligence not considered						
22. KEYWORDS (Proceed EACH with Security Classification Code) (U) Malaria; (U) Mosquitoes; (U) Trypanosomiasis; (U) Tsetse flies; (U) Immunization						
23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Proceed test of each with Security Classification Code.) 23. (U) Development of physiological means of interrupting malaria transmission through an understanding of factors affecting parasite infectivity in vivo and in vitro. Develop test systems for studying mechanisms underlying sporozoite induced immunity for the eventual prevention and control of malaria in military troops. Develop model for the transmission of African trypanosomiasis in the laboratory and for utilizing the parasites in studies involving the immune response.						
24. (U) Determine quantitatively such parameters as the minimum numbers of immune cells required to convey protection against Plasmodium berghei malaria. Isolation of different stages of the malaria parasite on density gradients for subsequent study in culture systems. Establish a self sustaining colony of tsetse flies followed by the cyclic transmission of trypanosomes between insect vector and rodent host. Screen various vertebrate and invertebrate cell cultures for the propagation and differentiation of the blood stages and insect cycle, respectively, of Trypanosoma rhodesiense.						
25. (U) 76 10 - 77 09 Considerable protection was afforded against sporozoite induced Plasmodium berghei malaria by the transfer of one million immune, unfractionated spleen cells and complete protection with 20 million cells. Cell fractionation studies indicated that immunity was transferred only with T cells and not with B cells. It has also been demonstrated that the immune mechanism is independent of macrophage function. Trypanosoma rhodesiense has been transmitted to tsetse flies but mature infections in the salivary glands have yet to be documented. Only one of eight cell systems thus far tested have supported the growth of Trypanosoma rhodesiense. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 76 - 30 Sep 77.						
*Available to contractors upon originator's approval. 1265						

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Project 3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT

Work Unit 082 Biological studies of insect infection and disease transmission

Investigators

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Description

The major objectives of this work unit have been (1) understanding the mechanisms underlying sporozoite induced immunity in Plasmodium berghei malaria, (2) the attempted establishment of a self-sustaining colony of tsetse-flies as a first step towards the development of a rodent/fly/rodent transmission cycle for Trypanosoma rhodesiense and (3) screening various primary and continuous cell cultures from both vertebrate and invertebrate species for the propagation and differentiation of the blood stages and the insect cycle, respectively, of a number of African trypanosomes.

Progress

1. The role of cell-mediated immunity in P. berghei malaria

Previous studies at WRAIR and at other institutions have shown that immunization of rodents with repeated injections of  $\gamma$ -irradiated P. berghei sporozoites results in extensive and often complete protection against a subsequent challenge of infectious, homologous sporozoites. Repeated exposure to irradiated sporozoites results in the development of anti-sporozoite antibodies, the sera of the immunized animals exhibiting both a sporozoite neutralizing activity which destroys sporozoite infectivity and a circumsporozoite precipitation reaction. Passive transfer of these antisera to normal recipients results in a reduction of the exo-erythrocytic forms but ultimately all recipients develop a parasitemia and die. The role of cell-mediated immunity in sporozoite induced immunity has been less extensively studied as initial attempts at adoptive transfer of immunity with sensitized spleen or peritoneal exudate cells were not successful. However, in more recent studies, undertaken with the collaboration of MAJ Wayne Hockmeyer and MAJ James Hall of the Armed Forces Institute of Pathology, significant progress has been made in defining the role of these cellular factors.

Balb/c mice, 8 - 10 weeks of age, were immunized against P. berghei malaria by one or more IV injections of irradiated sporozoites. Depending

on the experiment, the animals were either used after an initial injection of  $7.5 \times 10^4$  parasites or boosted one or more times with  $1 \times 10^4$  sporozoites. The standard immunization schedule was one initial injection followed by four boosters one week apart.

Cells for adoptive transfer procedures were obtained either from peritoneal exudates or from spleens of immune donors. Peritoneal exudates were induced by injecting 3 ml of light mineral oil 3 or 5 days before the cells were collected. Mice were killed by cervical dislocation and peritoneal exudate cells were collected by washing the peritoneal cavity with cold, buffered L-15 medium and collecting the cells with a sterile pipet after gentle massage. Spleens were removed from the animals under sterile conditions and minced with fine scissors. They then were gently pressed through a 60 gauge mesh into the harvesting medium. The cells were filtered through sterile gauze to remove any clumps and slowly drawn through a 26 gauge needle to insure a single cell suspension. The cells from several animals were pooled, washed 3 times and suspended in L-15 medium. Trypan blue was used to assess cell viability and the cells were counted in a Coulter counter after the red cells were destroyed by isotonic ammonium chloride.

Suspensions of spleen cells were fractionated into adherent and non-adherent cell populations using nylon wool. Both fractions were assayed for surface immunoglobulin (Ig) using fluorescein-conjugated rabbit antisera. The non-adherent cells were less than 10% Ig positive and are hereafter referred to as T-cells. The adherent cells were over 90% surface Ig positive and are referred to as B-cells. Following fractionation, cells were washed, resuspended in TC Medium 199 and counted.

Test cell suspensions, consisting of either non-immune control, immune unfractionated, immune T-cells or immune B-cells, and in varying quantities were transferred IV to recipient mice. The recipients were exposed to 600 rads in a  $^{60}\text{Co}$  gamma source 8 hours prior to cell transfer. These animals were then boosted 24 hrs later with  $10^4$  irradiated sporozoites intravenously.

To evaluate protective immunity, the recipient animals were challenged IP with  $10^4$  sporozoites. This dose produced infections in 98% of the control animals, all of which were lethal. Three days after challenge, Giemsa-stained blood films were examined daily for 10 days to determine the length of the prepatent period. Animals with prolonged prepatent periods compared to nonimmunized controls were considered to be partially immune. In certain groups of animals, the parasitemias were monitored until death. Cumulative mortality and parasitemia was monitored on all animals at 24 hours intervals. Immunized animals which did not develop an infection upon challenge were considered to be immune.

Initially, the ability of various quantities of immune unfractionated cells to convey protection was tested. In the first series of tests with the cell transfer system, one "spleen equivalent" or approximately 150 million cells were transferred to each irradiated recipient. However,

far fewer cells are required to transfer immunity as shown in Table 1. As few as  $10 \times 10^6$  immune cells offered complete protection against a sporozoite challenge while  $1 - 5 \times 10^6$  cells were sufficient to protect all but 3 out of 39 animals. Lesser numbers were not tested.

The results of the cell fractionation experiments are shown in Table 2. All of the animals receiving  $2.5 \times 10^6$  T-immune cells survived sporozoite challenge and 23 of 26 animals receiving  $0.5 - 1 \times 10^6$  were likewise protected. By contrast, all but one animal succumbed to infection after receiving  $1 - 2.5 \times 10^6$  B-immune cells. It is thus clear that protection is afforded solely by the transfer of immune T-cells, not of B-cells.

In one experiment, the role of macrophages in the immune response was evaluated by treating recipient mice with a macrophage-blockading agent (Carrageenan) for 14 days prior to challenge with sporozoites. The protocol followed is known to significantly reduce macrophage function. When such carregeenan treated mice served as the recipients in cell transfer experiments, the results were identical to those experiments using untreated recipients (see Table 3).

The duration of immunity transferred was studied by delaying the challenge until 4 weeks following cell transfer. In this experiment, mice which received  $10^6$  immune cells, or immune T- or B-cells were all protected. The protection observed in the B-cell transferred group was in all likelihood due to the opportunity for proliferation of a small number of T-cells present in the samples of transferred B-cells. Additional studies are underway to determine the upper limits of the duration of immunity.

## 2. Colony establishment of the tsetse fly, Glossina morsitans

Antigenic variation of Trypanosoma rhodesiense in the blood of a vertebrate host occurs indefinitely at 3-4 day intervals in such a way that immunogenicity specified by the original antigen does not confer protection against subsequently developed antigens. However, passage through the tsetse fly vector, Glossina morsitans, results in reversion to a basic or "parent" antigenic type (Gray, 1965) which predominates for approximately 3-4 days in the subsequent vertebrate infection as well. This suggests that metacyclic form trypanosomes from the fly vector can be used as immunogens to confer protection against challenge by the bites of other flies. To test this hypothesis, large numbers of uniformly reared tsetse flies must be infected with trypanosomes, which in turn must be transmitted to a vertebrate host. The emphasis of this research has thus been on the establishment of a self-sustaining colony of G. morsitans and the development of a model for the cyclical transmission of T. rhodesiense from rodent to rodent via tsetse flies.

A colony of tsetse flies was obtained in September, 1976 from the Tsetse Research Laboratory, College of Veterinary Science, University of

Bristol, England. Since that time, the colony has been maintained but only with the aid of a bimonthly input of tsetse puparia from the Tsetse Research Laboratory.

Initially, flies were given blood meals from guinea pigs. However, this procedure resulted in a decline in mean puparial weight from 27.4 mg in the parental generation to 25.7 mg in the F<sub>2</sub> generation (Table 4). Concurrently, the percent adult eclosion dropped from 95% (parental) to 68% (F<sub>2</sub>). When lop-eared rabbits became available (the host recommended by the Bristol workers), puparial weights and percent eclosion improved (28.6 mg and 93%, respectively). Unfortunately, these improvements were overshadowed by a reduction in adult longevity (Table 4) and a decreased fecundity. It thus became apparent that colony vigor was reflected by the four factors of puparial weight, fecundity, adult longevity and percent eclosion. The latter 3 measures can be collectively incorporated into one, viz., innate capacity for increase,  $\Gamma_m$ , and computed as follows:

$$\Gamma_m = \frac{\log_e(N_t/N_0)}{t}$$

where N<sub>t</sub> = number of individuals at time t

N<sub>0</sub> = number of individuals at time 0

t = time during which population growth is observed

On the basis of this formula,  $\Gamma_m$  for the parental generation was determined to be 0.0083 whereas the flies fed on the lop-eared rabbits had a  $\Gamma_m < 0$ . These figures compare very unfavorably with that obtained by the Bristol Laboratory.

The problem of feeding flies on lop-eared rabbits apparently developed due to the toxic effects of sulfatose (Jordan and Trewern, 1973) administered to rabbits to prevent the spread of coccidial disease under crowded conditions. Recently, the flies have been switched to a new host, New Zealand rabbits, which are less susceptible to this disease. Although final data are not yet available, improvement of puparial weights have been noted (Table 4) and adult longevity appears to have been increased.

In the near future, a further improvement is expected when flies can be maintained on defibrinated horse blood fed through a synthetic membrane. This procedure has been successfully employed for several years at Bristol and preliminary results with this membrane system have been encouraging.

### 3. Development of a cyclical transmission model

A strain of Trypanosoma rhodesiense which was obtained from a Kenyan in 1975 and syringe passed in rodents was used to obtain stabilates for immunological studies on antigenic variation as well as for studies on cyclical development. Trypanosomes from the first parasitemic peak in C57BL/6 mice were passed through a DE-52 (cellulose) column for purification, then resuspended in defibrinated horse blood to a titer of 2.0 - 2.5 x 10<sup>7</sup> trypanosomes/ml. This infected blood was fed through a

silicone membrane (maintained at 37°C) to teneral flies from 24 - 72 hrs post eclosion. Subsequently, selected flies were either dissected or fixed for histological preparation and examined for the presence of trypanosomes beginning on day 2 post-infection. Transmittal of the infection from fly to rodent was attempted by feeding the presumptively infected flies on mice beginning on day 14 post-infection. Such feeds on clean mice were continued on alternate days up to day 40-50 post-infection. The mice were observed for trypanosomes by preparing blood smears from the tail beginning 2 days after the infective feed and continuing on alternate days for 10 days. The regimen of tests conducted is shown in Table 5 and the results of these tests in Table 6.

The number of midgut infections obtained indicates how many initial infections survived in the fly. The number of established infections indicates the number of midgut infections which propagated successfully in the anterior midgut. The number of mature infections should indicate how many established infections produced infective (metacyclic form) trypanosomes in the salivary glands.

Since no mature infections developed in the salivary glands, and this was corroborated by not finding any infected blood from mice, several questions arose as to the nature of the problem. After tests 1 and 2, which were conducted in an uncontrolled environment having undesirably high temperatures (Harmsen, 1973), it was assumed that lowering the temperature would improve the infection (Jenni, 1977). This appeared to be the case in test 3 based on the number of established infections. However, the fact that still no mature infections were obtained suggested that the strain itself may be too far removed from the human host (13 passes) to retain its infectivity. This possibility was addressed in tests 4 and 6 where the trypanosomes had been passed 4 and 6 times, respectively. Unfortunately, 100% of the flies in test 4 died of unknown cause(s) within 8 days after the infective blood meal. But in test 6, despite the low passage number, none of the infections was mature as evidenced by the lack of infected mice. A second possibility is that insufficient numbers of flies were used in all of the above tests. A low level of susceptibility on the part of the flies plus a fairly high level of mortality during development in the vector may well require higher numbers of flies per test to detect mature infections.

#### 4. Cultivation of Trypanosoma congolense in a cell line of Glossina morsitans

T. congolense, the cattle pathogen, when grown in either blood-agar medium or in primary organ cultures derived from tissues of the tsetse fly vector, loses its infectivity for mammalian hosts after a few days of culture in vitro. The parasites undergo a transformation from the blood-stream form to procyclic culture trypomastigotes or midmastigotes which resemble the midgut forms in the tsetse fly. Subsequent development to the epimastigote and infective, metacyclic stage, as found in the salivary glands, has rarely been observed. The present study, in collaboration

with Drs. R. and E. Steiger and Dr. W. Trager of the Rockefeller University, was undertaken to determine whether a continuous line of G. morsitans cells would support the growth and development of T. congolense *in vitro*.

The cell line, initiated in 1973, was derived from a single third stage larva of G. morsitans and has been designated as WR73-Gm-1. The cells were grown in Schneider's Drosophila medium modified by the addition of 25 mM PIPES buffer. Conditioned medium was obtained by removing, centrifuging and filtering the supernatant from cultures with confluent monolayers.

Transformation of T. congolense (Strain TREU 1183) from the blood forms to the midmastigote and procyclic forms was completed within a few days of being placed in culture with either the Glossina cell line or in conditioned medium. Multiplication of the parasites was also roughly equivalent in both the cell line and conditioned medium, reaching a maximum density of  $4 - 6 \times 10^6/\text{ml}$  between days 6 and 8 following subculturing. By contrast, fresh medium supported the maintenance but not the growth of the parasites. Transition and epimastigote forms increased during the growth phases of the cultured cells and declined proportionally during the stationary phases. But all attempts to infect rats or mice with the cultured parasites were unsuccessful indicating that differentiation of the parasites *in vitro* ceases short of the metacyclic, infective stage. A more detailed report on the cultivation of T. congolense in this cell system is currently in press (Steiger et al., 1977).

##### 5. Cultivation of Trypanosoma rhodesiense in primary and continuous vertebrate cell cultures

Until recently, all attempts to culture the bloodstream forms of African trypanosomes *in vitro* resulted in a loss of their characteristic morphology and in their infectivity for vertebrate hosts. However, during the past year Hirumi et al., (1977) reported the successful cultivation of Trypanosoma brucei in a system employing a bovine fibroblast-like cell line and medium RPMI 1640 supplemented with 20% fetal bovine serum. The trypanosomes so grown were morphologically indistinguishable from the bloodstream forms and retained their infectivity for 220 days at 37°C. The use of a mouse L-cell line and a dog kidney cell line were also adequate to maintain trypanosomes which had previously been adapted to culture with the bovine fibroblast-like line but were far inferior to the latter if used to initiate cultures with bloodstream forms taken directly from the donor. The potential of this system for obtaining large numbers of parasites for vaccine purposes, for biochemical studies, for insight into the mechanism of antigenic variation is readily apparent and the purpose of the current research was to test the possibility of using the same or a similar culture system to grow the bloodstream forms of T. rhodesiense.

A Wellcome strain (CP3B5) of T. rhodesiense, maintained in ICR mice, was used throughout these experiments. Prior to the peak in parasitemia, the blood was aseptically withdrawn by heart puncture and mixed 1:1 with

a cold balanced salt solution. The trypanosomes were separated from the blood either by density gradient centrifugation using Ficoll-Hypaque (density 1.077) or with a rabbit anti-mouse red blood cell preparation. The numbers of trypanosomes were adjusted to give an initial concentration in each culture flask of approximately  $0.5 \times 10^5/\text{ml}$ .

Considerable difficulty was experienced in obtaining a bovine fibroblast-like line from buffy coat cells. Of 28 primary cultures initiated at approximately weekly intervals, only 2 thus far have had sufficient numbers of monocytes, which may be the cells Hirumi and his colleagues refer to, to insure some success at subculturing. (An alternative and perhaps even more likely explanation regarding the fibroblast-like line is that it originated from cells accidentally scrapped from the lining of the blood vessel during insertion of the hypodermic needle). In the absence of such cultures, a number of primary, early passage and continuous vertebrate lines were assayed for their suitability as a substrate for the growth of T. rhodesiense. The cultures used were as follows:

Primary or early passage cultures

- 1) Canine peritoneum (supplied by LTC E. H. Stevenson, Division of Veterinary Resources)
- 2) Chick embryo
- 3) African Green Monkey Kidney (supplied by Mr. D. Dubois, Department of Bacterial Immunology)

Diploid lines

- 1) WI-38 (human embryonic lung)

Heteroploid lines

- 1) L929 (mouse connective tissue)
- 2) BHK (baby hamster kidney)
- 3) VERO (African Green Monkey kidney)
- 4) LLC (Rhesus Monkey kidney)

According to Hirumi et al, (1977) 3 main conditions must be met if the trypanosomes are to retain their bloodstream characteristics during in vitro culture, namely: the density of the trypanosomes in both the original and successive subcultures should be kept at  $1 - 5 \times 10^5/\text{ml}$ ; the cultures must be initiated with a trypanosome population in which the great majority of the parasites are long and slender in form, i.e., from a rising parasitemia; a suitable cell substrate (preferably a bovine fibroblast-like line) must be used with RMPI 1640 medium and 20% fetal bovine serum. Only the second of these conditions could be routinely satisfied. Controlling the trypanosome population, with respect to both density and morphological characteristics ranged from impossible to difficult depending upon the cell substrate employed. Only the L929 cells showed any promise of supporting the growth of T. rhodesiense for extended periods of time without loss of characteristic morphology or infectivity

(Table 7). The other cell substrates were adequate for less than 24 to no more than 120 hrs.

Conclusions and recommendations

1. The results from the cell transfer studies clearly indicate that the mechanism of sporozoite-induced immunity in *P. berghei* infections requires T lymphocytes and is completely operative in the absence of B lymphocyte or macrophage function. The most plausible explanation for the mechanism is either direct T-cell killing of sporozoites or the elaboration of a soluble factor (lymphokine) by the T-cells which is lethal to the sporozoites. Emphasis should be directed toward in vitro studies, the results of which may distinguish between the two alternatives.

2. A self sustaining colony of tsetse flies has not yet been achieved. The use of New Zealand rabbits and perfection of the synthetic membrane system of feeding appear to be the most promising means of increasing colony numbers and vigor.

3. Mature infections of *T. rhodesiense* in *G. morsitans* flies have yet to be documented. To insure a greater probability of success, the number of flies per test should be increased, the flies should be fed directly on infected mice on 2 successive days to bracket a low percentage of stumpy forms (20-40%), an uncloned isolate (LVH-18) with no more than 4-6 passes from the human host should be used to improve trypanosome adaptability to the flies and the post infection temperature should be held at 20°C for 4 - 6 hours (to enhance trypomastigote transformation) followed by a 26°C holding temperature to accelerate trypanosome propagation and development. A more recent human isolate of *T. rhodesiense*, preferably one known to have passed recently through a fly, might also be obtained and tested as above.

4. Blood forms of *T. congolense* will transform in the presence of a continuous cell line of *G. morsitans* and the procyclics formed can be propagated continuously. However, although midmastigotes readily differentiate into epimastigotes, development stops short of the infective, metacyclic stage. The *Glossina* cell line may not be capable of supporting the entire insect stages of the parasites due to the long adaptation time it required to adjust fully to the culture conditions. The cells which now comprise the line have inevitably undergone considerable selection during the interval of adaptation. Primary cultures or such cultures with the cell line serving as a feeder layer might result in further differentiation on the part of *T. congolense*.

5. Of some 8 different primary, early passage and continuous vertebrate cell cultures assayed for their suitability to support the blood-stream forms of *T. rhodesiense*, only the L929 line, derived from mouse connective tissue, showed any promise. Efforts should be made to monitor such cultures more closely, i.e., 3 rather than 2 times per 24 hrs to ensure that the density of the trypanosome population does not exceed

$5 \times 10^5$ /ml. The bovine cell cultures, obtained from buffy coat preparations, should be tested as soon as they are capable of withstanding subcultivation. Subcultures of both the ILR-BPF-376 and ILR-BPF-476 lines used by Hirumi et al. (1977) to successfully grow the bloodstream forms of T. brucei have been requested as will be tested on T. rhodesiense as soon as possible.

Project 3M762770A803, MALARIA PROPHYLAXIS AND TREATMENT

Work Unit 082 Biological studies of insect infection and disease transmission

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TABLE 1

Numbers of immune cells required to transfer protection against  
P. berghei malaria

Status of cells	No. x 10 <sup>6</sup>	Parasitemia/total mice
Non - immune	200	8/8
	100	4/5
	40	10/10
	20	7/9
Immune	200	0/8
	100	0/10
	40	0/16
	20	0/31
	10	0/21
	5	2/18
	2.5	0/8
	1	1/13

TABLE 2

Type of immune cells required to transfer protection against  
P. berghei malaria

Source/statue of cells	No. x 10 <sup>6</sup>	Parasitemia/total mice
Normal	2.5	9/9
	1.0	6/6
Immune	2.5	0/8
	1.0	1/13
T-immune	2.5	0/8
	1.0	1/16
	0.5	2/7
B-immune	2.5	2/2
	1.0	5/6

TABLE 3

**Effect of Carregeenan on transfer of immunity against P. berghei  
malaria**

Status of cells transferred	No. $\times 10^6$	Recipient	Parasitemia/total mice
Normal	1	Untreated	6/6
Normal	1	Cg. treated	6/6
Immune	1	Untreated	0/6
Immune	1	Cg. treated	0/6
T-immune	1	Untreated	1/6
T-immune	1	Cg. treated	0/6

TABLE 4  
Summary of tsetse fly colony data, compared by host animal

Host	Fly Generation	Mean No. puparia per Female	Mean puparial weight (mg) I S.E.	% Adult Eclosion (>100 puparia)	Mean Adult Longevity (Days)	Innate Capacity for Increase ( $R_m$ )
Guinea Pig	Parental	3.9	27.4 ± 0.2	95	109	0.0083
Guinea Pig	F <sub>1</sub>	2.25	26.2 ± 0.4	70	-	-
Guinea Pig	F <sub>2</sub>	-	25.7 ± 0.6	68	-	-
Lop-Eared Rabbit	F <sub>1</sub>	1.4	28.6 ± 1.0	93	59	<0
New Zealand Rabbit	Parental	-	29.6 ± 1.0	-	-	-

TABLE 5

Detailed history on clones/populations used to infect tsetse flies in 6 different tests

<u>Test #</u>	<u>Clone population</u>	<u>Passage number</u>	<u>Infecting temperature</u>	<u>Percent viable</u>	<u>Percent stumpy</u>
1	WRATat 3p7	13	33C	23	20
2	WRATat 3p7	13	35	90	25
3	WRATat 2p6	13	25	70	60
4	LVH 18-3→4	4	23	-	-
5	WRATat 2p6	13	23	96	40
6	LVH 18-5→6	6	21	82	79

TABLE 6

Results of 6 different T. rhodesiense infections shown in Table 1

<u>Test #</u>	<u>No. of flies fed</u>	<u>No. of flies examined</u>	<u>No. of midgut infections</u>	<u>No. of established infections</u>	<u>No. of mature infections</u>
1	42	15	5	1	0
2	21	5	0	0	0
3	42	8	2	2	0
4	42	100% mortality within 8 days, no data			
5	65	4	0	0	0
6	34	None examined to date			

TABLE 7

Ability of different vertebrate cell substrates to support the growth of Trypanosoma rhodesiense in vitro. Data based on minimum of 3 runs containing 4 cell cultures each

Cell substrate	Max. time in culture (hrs)	Retention of infectivity to mice	Transformation from lg, slender to "stumpy" forms	No. of subcultures attempted
LLC	24	<24 hrs	<24 hrs	0
Canine peritoneum	72	24 hrs	24 hrs	0
VERO	72	NT	24-48 hrs	0
African Green monkey kidney	96*	NT	24-48 hrs	0
BHK	96*	NT	24-48 hrs	0
Chick embryo	96*	48-72 hrs	48-72 hrs	0
WI-38	96-120	72 hrs	48-96 hrs	1
L929	5 wks	1-4 wks	1-4 wks	4-14

\* But obviously in poor condition as judged by morphology and motility

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>2</sup> DA OC 6435	2. DATE OF SUMMARY <sup>3</sup> 77-10-01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY 76-10-01	4. KIND OF SUMMARY D. Change	5. SUMMARY SECY <sup>4</sup> U	6. WORK SECURITY <sup>5</sup> U	7. REGADING <sup>6</sup> NA	8. ORIGIN INSTN <sup>7</sup> NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES: <sup>8</sup> a. PRIMARY 62770A	PROGRAM ELEMENT 3M762770A803	PROJECT NUMBER 00		TASK AREA NUMBER 083	11. LEVEL OF SUM- A. WORK UNIT		
b. CONTRIBUTING CXXBXNMNMDPDX	CARDS 114F						
11. TITLE (Proceed with security Classification Code) <b>(U) Protective Immunity in Protozoan Diseases</b>							
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>9</sup> <b>002600 Biology 010100 Microbiology</b>							
13. START DATE 74 07	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY	16. PERFORMANCE METHOD C. In-House			
17. CONTRACT/GANT	18. RESOURCES ESTIMATE FISCAL YEAR		19. PROFESSIONAL MAN YRS 77	20. FUNDS (in thousands) 218			
a. DATES/EFFECTIVE: NA b. NUMBER: <sup>10</sup> c. TYPE: d. KIND OF AWARD:	EXPIRATION: e. AMOUNT: f. CUM. AMT.		78	5.0 637			
21. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research ADDRESS: Washington, DC 20012	22. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Div of CD&I ADDRESS:						
RESPONSIBLE INDIVIDUAL NAME: Garrison Raptund, COL TELEPHONE: 202-576-3551	PRINCIPAL INVESTIGATOR (Furnish DOD if U.S. Academic Institution) NAME: Carter L. Diggs TELEPHONE: 202-576-3544 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]		ASSOCIATE INVESTIGATORS NAME: [REDACTED]				
23. GENERAL USE Foreign intelligence not considered	24. KEYWORD (Proceed EACH with Security Classification Code) <b>(U) Antigens; (U) Protozoa; (U) Immunity; (U) Tropical Medicine; (U) Antibodies</b>		25. TECHNICAL OBJECTIVE, <sup>11</sup> 26. APPROACH, 27. PROGRESS (Furnish individual paragraphs identified by number. Proceed each with Security Classification Code.)				
23 (U) The objective of this work unit is to elucidate the protective mechanisms involved in immunity to malaria and African sleeping sickness. Malaria is a disease which has repeatedly impeded military operations, and African sleeping sickness has a high potential for doing so should there be troops in the endemic area. Studies of sleeping sickness are being added to this work unit for FY 78; they were included in work unit 202 during FY 77.							
24 (U) The approach used in these studies is to study both in animal models and through the use of the in vitro techniques the response elicited by the parasites on the immune system, to determine the roles of cellular and molecular mediators in these processes, and to design experimental immunogens which will provide the basis for future vaccine development programs. Malaria studies will emphasize the use of rodent models; most work with human malaria parasites will be conducted in work unit 098 and elsewhere.							
25 (U) 76 10-77-09 The culture technique developed in this laboratory has been used to analyse the properties of the human erythrocyte receptor for Plasmodium falciparum. A highly efficient assay of P. falciparum growth using $^{3}H$ -hypoxanthine incorporation has been developed and is being used to develop systems for the evaluation of the effects of drugs and antibodies. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1976 - 30 September 1977.							
1284							

Available to contractors upon originator's approval.

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  
Work Unit 083 Protective immunity in protozoan diseases

Investigators: COL Carter L. Diggs, M.D., MAJ David Haynes, M.D.  
and MAJ Jeffrey Chulay, M.D.

Assistants: Barbara Flemmings, B.S.; Andre Toussaint, B.S., M.S.;  
James Dillon, B.S.; SP4 Karen Czarnecky, B.S., SP6  
Barry Ellis, B.S., SP5 Cynthia Hall, B.S.

Objective: The objectives of this project are to improve the *in vitro* culture of the human malaria parasite Plasmodium falciparum and to use the cultured parasite in immunologic and biochemical studies elucidating the host-parasite relationship. The eventual goal is to evaluate the feasibility of a human malaria vaccine.

Description: The long-term culture of the human malaria parasite Plasmodium falciparum was not possible until recently (1, 2). Our publication this year was the result of work completed last year (1). The recent description of a malaria vaccine made from P. knowlesi parasite merozoites, which protects monkeys against this species of monkey malaria parasite (3), has provided impetus to carry out similar studies with the human malaria parasite P. falciparum, which is responsible for an estimated one million deaths a year in Africa alone (4). Now that the parasite can be cultured, it is hoped that soon it can be obtained in the quantity and purity necessary to perform the antigenic analyses which might lead to an effective vaccine. The Aotus monkey has been chosen as the animal model best suited to the initial evaluation of the immune response to P. falciparum. Additional studies, which can be done even without further improvements in culture, but are also of great potential significance, use the parasite culture to screen anti-malarial drugs (and evaluate their mode of action, thus aiding the further rational development of anti-malarials); and to describe the interactions of the parasite with human red cells, for example red cells with sickle hemoglobin or a deficiency of the enzyme G6PD, both of which are thought to confer some degree of protection against the parasite.

Progress: Our group has been fortunate to acquire new personnel and a new laboratory in July 1976. The inevitable disruption to the flow of research has now passed, and we are again evaluating new culture techniques, applying the presently available methods to some problems, and beginning a program of immunological evaluation of the protective immunity afforded Aotus monkeys following chemotherapeutic cure of infection with P. falciparum.

One of the first uses made of the culture was an evaluation of the differences in erythrocyte surface receptors for P. falciparum and P. knowlesi. Dr. Louis Miller (NIAID, NIH) had previously shown that

the monkey malaria parasite P. knowlesi had characteristics similar to the human malaria P. vivax in that both required the presence of the red cell surface antigen known as "Duffy" in order to invade that red cell. The Duffy antigen is genetically absent from the red cells of most of the Northern African population, conferring absolute resistance to infection with P. vivax. Furthermore, treatment of a Duffy positive red cell with chymotrypsin (but not trypsin) seems to remove the receptor which the P. knowlesi parasite recognizes (requires for invasion). These studies were repeated in collaboration with Dr. Miller, this time comparing the red cell invasion requirements of P. knowlesi and P. falciparum (5). The results presented a marked contrast between the two parasites; the presence or absence of the Duffy antigen had no effect on invasion by P. falciparum. Also, treatment of the red cells with trypsin but not chymotrypsin inhibited invasion by P. falciparum --- the converse of what was found with P. knowlesi in the same experiment (table 1). Of several human rare blood cell types tested, only one, "En (a-)", which is deficient in one of the surface glycoproteins showed reduced invasion by P. falciparum. Work in progress will use purified red cell glycoproteins to further characterize this parasite - red cell interaction. It is hoped that this might lead to a means of isolating the parasite surface molecule(s) that interact with the red cell surface. This might be a good source of antigen for vaccine development since this parasite- red cell interaction is crucial to the survival of the parasite and might be blocked by antibody. (Antibody to the parasite has been thought to play an important role in protective immunity in some experimental models of malaria (6).

Our present culture technique derives from our previous work (1), combined with the technique described by Trager and Jensen (2), with some further minor modifications: we are culturing the parasite in sealed tissue culture flasks containing human 6% H+ red cells and a media of RPMI 1640 with added HEPES buffer (15 mM) and 10% fresh frozen plasma, gassed with 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% nitrogen. Dilution are made into fresh red cells every few days. Using no antibiotics, but a meticulous technique (somewhat painful acquired) allows continuous culture for months without infection. During our early experience we found that using media made up freshly from powder, rather than liquid as supplied by the manufacturer, allowed an increased growth rate from 2 fold every 48 hr. cycle (using either M199 or RPMI 1640) to 4 fold or more every cycle. Because fetal bovine serum proved unsuitable for sustaining long-term cultures, and human serum is difficult to obtain in large quantities, we tried using human fresh frozen plasma, obtained from the WRAMC blood bank (LTC Radcliffe). When first heat-inactivated and the precipitated fibrinogen removed, this plasma was shown to be a satisfactory replacement for human serum, and has the distinct advantage of not destroying the red blood cells, which has been separated from the plasma and are available for transferring patients. In one 9 day experiment comparing plasma and serum, the two supported growth as evaluated by morphologic increase in numbers of parasites about equally well (2,011 fold increase and 1,267 fold increase respectively). However, when evaluated by the criteria of 3H-hypoxanthine incor-

poration into polynucleotides, the plasma was much superior 43 thousand cpm vs. 14 thousand cpm for serum, using 50  $\mu$ l samples of culture pulsed with 0.5  $\mu$ Ci 3H-hypoxanthine in quadruplicate microtiter wells incubated 18 hours, then harvested on to glass fiber filter paper using a multiple automated sample harvester). It is likely that purine nucleotides present in serum, but not plasma, competed with the radio-labelled hypoxanthine for uptake by the parasite. Parasites taken from continuous culture have been incubated with various dilutions of anti-malarial drugs prepared by automatic serial two fold dilutor in microtiter plates, then pulsed with 3H-hypoxanthine overnight and then harvested on to glass filters (done in collaboration with MAJ. Desjardins, Department of Pharmacology). Inhibition of uptake has been observed with nanogram per ml quantities of drug. Effective doses for 50% inhibition of incorporation of the hypoxanthine into RNA and DNA can be calculated using a paper punch output from the scintillation counter fed into a Tektronix 4051 graphics computing system which does a regression analysis. Four drugs may be evaluated in one plate, tested in triplicate wells, over a 64-fold concentration range. In addition to drug screening and bioassay of drug levels in human plasma, this system may prove helpful in elucidating mechanisms of drug action on the parasite if time-course studies are done on polynucleotide synthesis, and also protein synthesis is simultaneously evaluated by incorporation of radiolabelled leucine (initial studies suggest that this is feasible).

There are several obstacles to the purification of parasite antigen for immunologic studies. Those of most immediate and obvious importance are (a) the limit of 5 to 10% peak parasitemia in long-term cultures, (b) the need for large amounts of human plasma, if large amounts of parasite are to be cultured, (c) the asynchrony of the culture, (d) the necessity of frequent changes of culture medium if the highest parasitemias are to be obtained. Attempts to remove uninfected red cells by decreasing their total number have thus far not significantly increased peak parasitemia rates -- the total number of parasites supported in that culture also decreased, suggesting that the uninfected red cells supply some nutritive or other environmental factor to the parasite. Work with red cell lysates has met with some initial success in reducing plasma concentration requirements, and may prove to be of some use in obtaining higher parasitemia rates. Other constituents known to be present in plasma or red cells, but absent from RPMI 1640, are also being tested in attempts to overcome these obstacles. The synchronization of the culture, so that the stage of the parasite to be obtained for analysis may be selected, is in a very preliminary stage, using cycles of elevated incubation temperature. A continuous flow chamber has been fabricated (WRAIR Instrumentation). Preliminary testing indicated the need for better tubing connections, which have been ordered.

The poor morphologic appearance of the merozoite form of the parasite obtained from these cultures suggest some breakdown of antigenic structure. This is a problem which can be better investigated once the obstacles described in the preceding paragraph have been overcome.

Based on earlier work at WRAIR which showed that Aotus monkeys could be immunized against further challenge by one or two infections with *P. falciparum*, and that this immunity could at least in part be transferred by giving immune serum to non-immune monkeys (7), we are repeating the study to improve the statistical analysis, and to allow a more detailed analysis of the development of the protective immune response, as evidenced by changes from pre- and post-infection in vitro assays of the immune response. Four monkeys have already been immunized by infection and drug cure. Pre- and postinfection (immunization) plasma and lymphocytes are being collected from the the monkeys will be cryopreserved and will be compared simultaneously at a later date. In vitro assays to be used will include examination of plasma (and purified antibody) for blocking of parasite growth, and the stimulation of lymphocytes by parasite antigen to incorporate  $^{3}\text{H}$ -thymidine and to produce chemotactic lymphokines. Human immune serum has been shown to block parasite growth in vitro (8). We will look at blocking of parasite growth by immune Aotus plasma, and also examine plasma using an IFA test that we have developed for anti-parasite antibody using parasites obtained from culture. We have isolated small amounts of crude parasite antigen (merozoite isolation as described in reference 1) and demonstrated stimulation of immune Aotus lymphocytes as compared with non-immune lymphocytes (three fold increase of  $^{3}\text{H}$  thymidine incorporation). Dr. Wyler (NIAID, NIH) has agreed to examine the supernatants from stimulated lymphocytes for the presence of a chemotactic lymphokine, which he thinks plays an important part in the immune response to malaria (9).

TABLE I

The Effect of Enzyme Treatment of Human Erythrocytes  
on Invasion by P. falciparum and P. knowlesi

<u>Enzyme</u>	<u>P. falciparum</u>	<u>P. knowlesi</u>
Chymotrypsin, 0.1 mg/ml	-2.3 $\pm$ 4 (3)	90 (1)
Neuraminidase, 25 U/ml	54 $\pm$ 8 (6)	-9 $\pm$ 21 (3)
Trypsin, 1 mg/ml	78 $\pm$ 3 (6)	2.5 $\pm$ 11 (4)
Trypsin, plus neuraminidase	93 $\pm$ 2 (2)	26 $\pm$ 15 (2)
*Percent reduction = $\frac{(\text{percent infection of enzyme-treated cells} - \text{percent infection of control cells})}{\text{percent infection of control cells}} \times 100$		

The resistance of chymotrypsin (0.1 mg/ml) treated erythrocytes to invasion by P. knowlesi in one parallel experiment corresponded to the results of published data for six experiments under similar conditions (95  $\pm$  2.5 ) (SD) percent reduction in invasion). P 0.01 Comparison of invasion of control and enzyme-treated erythrocytes by the paired t test.

Project 3M762770A803 MALARIA PROPHYLAXIS AND TREATMENT  
Work Unit 083 Protective immunity in protozoan diseases

Literature Cited.

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1. Haynes, J.D., Diggs, C.L., Hines F.A., and Desjardins, R.E. Culture of human malaria parasites Plasmodium falciparum. Nature 263: 767, 1976.
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9. Wyler, D.J., and Gallin, J.I.: Spleen-derived monocellular cell chemotactic factor in malaria infections: A possible mechanism for splenic macrophage accumulation. J. Immunol. 118:478, 1977.

Publications:

1. Haynes, J.D., Diggs, C.L., Hines, F.A., and Desjardins, R.E. Culture of human malaria parasites *Plasmodium falciparum*. *Nature* 263. 767, 1976.
2. Miller, L.H., Haynes, J.D., McAuliffe, Shiroishi, T., Durocher, J.R., and McGinnis, M.H.: Evidence for differences in erythrocyte surface receptors for the malarial parasites, *Plasmodium falciparum* and *Plasmodium knowlesi*. *J. Exp. Med.* 146: 277, 1977.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>b</sup> DA OB 6495	2. DATE OF SUMMARY <sup>b</sup> 77 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMMARY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY <sup>a</sup> U	6. WORK SECURITY <sup>a</sup> U	7. REGRADING <sup>b</sup> NA	8. DESIGN INSTRN <sup>b</sup> NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES <sup>a</sup> b. PRIMARY 72770A	PROGRAM ELEMENT PROJECT NUMBER 3M762770A803			TASK AREA NUMBER 00	10. LEVEL OF SUM- A. WORK UNIT WORK UNIT NUMBER 084	
c. CONTRIBUTING C. CONTRIBUTING CARDS 114F						
11. TITLE (Pecede with Security Classification Code) <b>(U) Synthesis of Antimalarial Drugs</b>						
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>a</sup> 012100 Organic Chemistry						
13. START DATE 72 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA		16. PERFORMANCE METHOD C. In-House		
17. CONTRACT/GRAANT		18. RESOURCES ESTIMATE		19. FUNDING AGENCY		
20. DATES/EFFECTIVE: b. NUMBER: c. TYPE: d. KIND OF AWARD:		EXPIRATION:		FISCAL YEAR	A. PROFESSIONAL MAN YRS 77	B. FUNDS (In thousands) 450
				CURRENT	7.0	512
21. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research ADDRESS: Washington, DC 20012		22. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Division of Medicinal Chemistry ADDRESS: Washington, DC 20012		23. PRINCIPAL INVESTIGATOR (PUNISH SEAN II U.S. Academic Institution) NAME: Sweeney, T.R., PhD TELEPHONE: 202/576-3731 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]		
24. GENERAL USE Foreign intelligence not considered		25. ASSOCIATE INVESTIGATORS NAME: Canfield, C.J., COL NAME: [REDACTED]				
26. KEYWORDS (Pecede EACH with Security Classification Code) <b>(U) Malaria; (U) Drug Development; (U) Antimalarials; (U) Chemical Syntheses</b>						
27. TECHNICAL OBJECTIVE. <sup>a</sup> 28. APPROACH. 29. PROGRESS (Punish individual paragraphs identified by number. Pecede last of each with Security Classification Code.)						
23. (U) The objective is to manage, integrate, and provide technical direction for both a contract and in-house program to obtain potentially active antimalarial compounds for military use through rational organic syntheses.						
24. (U) Necessary research areas are defined, proposed research evaluated, ongoing research guided, evaluated, and integrated with the other program elements. Technical advice is obtained through an Ad Hoc Study Group on Medicinal Chemistry. Information is exchanged by contractors through technical meetings.						
25. (U) 76 10 - 77 09 The development of curative and prophylactic antimalarials continued to receive primary attention during the year. A class of compounds has been developed that shows very high curative activity against a sporozoite-induced relapsing monkey malaria, much better than primaquin as well as high curative activity against non relapsing malaria. A large batch of this compound is being prepared for preclinical studies. Three other new classes of compounds showing very high activity against trophozoite-induced malarial infections have also been uncovered. Candidates for advanced study will soon be selected. Many new 2-acetylpyridine 4-substituted thiosemi-carbazones were synthesized for antimalarial testing and structural requirements for activity. Derivatives of 4-amino-2-methoxy-9-methylacridine as candidate antimalarials are being synthesized. The rational synthesis program produced about 422 new compounds over the reporting period; about 306 of these were target compounds; target compounds from the in-house program are included. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.						
1292						

<sup>a</sup>Available to contractors upon originator's approval.

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M76270A803 MALARIA PROPHYLAXIS

Task 00

Work Unit 084 Synthesis of Antimalarial Drugs

Investigators:

Principal: Thomas R. Sweeney, Ph.D.

Associates: COL Craig J. Canfield, MC; June A. Page, M.S.; Bing T. Poon, Ph.D.; Daniel L. Klayman, Ph.D.; CPT John P. Scovill, Ph.D.; Edgar A. Steck, Ph.D.; Richard E. Strube, Ph.D.

The Research Contract Chemical Synthesis Program

1. Malaria

During this reporting period there were 11 active synthesis contracts; it is expected that 8 of these will be carried over in FY-78. The Medicinal Chemistry Study Group approved 6 proposals which is expected to result in 4 new contracts to start 1 October 1977. Five patent applications are in various stages of processing.

Contract research areas that were discontinued during the period either because of lack of activity of the target compounds or an inability to improve the activity of active compounds include naphthalene analogs of 8-aminoquinolines, 1,5-naphthyridines, 1,2,4,5-tetrazines and related triazines, phenazine derivatives and a variety of types of substituted pyrimidines.

Continuing research areas include those that are based upon biochemical rationales. These include the synthesis of compounds designed to interfere with parasite phospholipid synthesis, compounds designed to inhibit the enzyme hypoxanthine phosphoribosyltransferase and compounds designed to inhibit thymidylate synthetase.

From the standpoint of uncovering highly active classes of compounds, the contract synthesis program has enjoyed an outstandingly productive period. Four new classes of compounds have emerged, each of which will very likely produce at least one compound for clinical study. These are the 5-aryloxy-6-methoxy-8-aminoquinolines, new Mannich bases related to amodiaquine, acridine diones and imine derivatives of the acridine diones. Work in these areas is being pursued vigorously.

2. Leishmaniasis

The Study Group approved 2 proposals for the synthesis of new anti-leishmanial agents and it is anticipated that they will start at the

beginning of FY-78. Both are in the area of 8-aminoquinolines and both are based upon developing two compounds that showed outstanding activity.

### 3. Trypanosomiasis and Schistosomiasis

No synthetic programs have been started in these areas.

#### The Preparations Laboratories

The two preparations laboratories are used chiefly to resynthesize large quantities of selected compounds that are needed for clinical, pharmacological, or large animal studies. On occasion they may make a large quantity of an intermediate that would be generally useful in the synthesis program.

The output of the preparations laboratories is summarized in the following table. It should be recognized that some of the compounds received may have been requested in an earlier reporting period and some requested in this period will not be received until the next period.

FY-7T and -77	Target Compounds				Total
	>1000	100-1000	<100	Intermediates	
Number requested	3	5	18	10	36
Number received	1	1	14	10	26

#### The Analytical Laboratory

The analytical laboratory is used to check the identity and purity of bulk and formulated drugs and to determine the stability (shelf life) of formulated drugs. The following table summarizes the reports from the laboratory.

#### Analyses

	Bulk	Formulations	Stability Determinations	Special
Requested	8	21	23	49
Received	10	17	17	--

#### Acquisition of Compounds

The following table summarizes the number of various classes of compounds

received during FY-7T and -77.

	<u>Originals</u>	<u>Duplicates</u>	<u>Total</u>
Purchased	188	12	200
Gifts	247	97	344
Synthesized	629	101	730
Discreet	4605	353	4958
Prep labs	48	45	93
Total	5717	608	6325

Twenty-two companies submitted compounds under the no-dollar agreement during the reporting period. Six new agreements were signed, two were cancelled and four are under negotiation.

#### Data Processing

##### 1. Inventory

The new inventory system is in final stages of check-out. Documentation is being prepared. This system is due to start parallel running with the existing system on 1 November. After one month of successful parallel testing the old system will be discontinued.

##### 2. Chemistry

All programs for the creation, update, and maintenance of the chemistry files have been completed. File building has begun with about 17,000 structures being added to the new data base. A serious error in the program that generates the screens was detected at this point and further data base building suspended until the correction can be made and its impact analyzed. The on-line whole structure identity search has been completed. Programs for the sub-structure search and the integrated search system are being written.

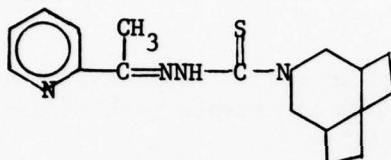
Contractual efforts have been integrated with those in-house. Work began on the on-line editing and correction of chemical structures for data base building. An Imlac graphics terminal was installed at the downtown facility. Recommendations for improving the area where chemicals are handled have been made and will be implemented during FY-78.

#### Organic Synthesis Laboratory

During the past year an additional 66 2-acetylpyridine 4-substituted thiosemicarbazones were synthesized and submitted for antimalarial testing. It was observed that various substituents such as bromo or nitro on the aromatic or benzyl ring in the 4-position of the thiosemicarbazone destroy activity whereas other groups such as chloro, methyl, or methoxy enhance activity.

Semicarbazones, related to active thiosemicarbazones, have been clearly demonstrated to be devoid of activity. Thus, the essentiality of the sulfur atom has been established.

The most important improvement in activity in the thiosemicarbazone series has been achieved by incorporating the nitrogen atom at the 4-position into a cyclic system such as a piperidine ring. One of the most potent examples of such a compound is WR 230,190 which cured test animals at a dose level as low as 20 mg/kg. This is a striking



improvement in the potency of the thiosemicarbazones over the situation which existed a year ago when the best in the class gave cures at 320 mg/kg.

$\text{Fe}^{++}$  Metal chelates of some active thiosemicarbazones, prepared utilizing  $\text{Cu}^{++}$ , are now being evaluated.

Four randomly-chosen 2-acetylpyridine thiosemicarbazones examined in the Smith-Genthaler antifol bioassay showed marked inhibition of the three bacterial strains used. These observations are being followed up by the Division of Communicable Disease and Immunology, WRAIR, which is currently screening two 2-acetylpyridine thiosemicarbazones against a broad spectrum of bacteria.

A preliminary synthetic route has been developed leading to 4-amino-2-methoxy-9-methylacridine. This is low yield sequence which requires improvement. When a sufficient quantity of the acridine has been made, the amino function will be substituted with various groups which impart antimalarial activity.

Twenty samples of various kinds of wood from Brazil donated to us by COL Radke, after having been reduced to a powder courtesy of the U.S. Forestry Service (Madison, WI), were treated with hot methanol-chloroform over an extended period of time. The extracts obtained, which range in appearance from a fine powder to a dark, heavy gum, have been submitted for testing in the WRAIR schistosomiasis screen.

The isolation and determination of primaquine, its analogs and metabolites in buffer solutions has been investigated using various analytical techniques. In particular, ir, uv, NMR, mass spectrometry, as well as TLC, have been evaluated for the applicability to future metabolism studies. The methodology which has evolved will shortly be applied to the assay of primaquine and its metabolites in biological fluids.

In the past year the following new instrumentation has been put into operation: a Finnigan 31000 Gas Chromatograph/Mass Spectrometer; a Varian T60-A Nuclear Magnetic Resonance Spectrometer; and a Hewlett-Packard 5840A Gas Chromatograph.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>a</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL
				DA OB 6535	77 10 01	DD-DR&E(AR)636
1. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SECY <sup>c</sup>	6. WORK SECURITY <sup>c</sup>	7. REGRADING <sup>d</sup>	8. DISIN' INSTRN	9. SPECIFIC DATA-CONTRACTOR ACCESS
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: <sup>e</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	11. LEVEL OF SUM	
B. PRIMARY	62770A	3M762770A803		00	12. WORK UNIT NUMBER	
B. CONTRIBUTING						
C. CONTRIBUTING	CARDS 114F					
11. TITLE (Pencode with Security Classification Code) <sup>f</sup>						
(U) Biological Evaluation of Antimalarial Drugs						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>g</sup>						
012600 Pharmacology 002600 Biology						
13. START DATE	14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY	16. PERFORMANCE METHOD		
66 07	CONT		DA	C. In-House		
17. CONTRACT/GANT	NA		18. RESOURCES ESTIMATE			
B. DATES/EFFECTIVE:	EXPIRATION:		FISCAL	19. PROFESSIONAL MAN YRS	20. FUNDS (in thousands)	
B. NUMBER:			YEAR	77 CURRENCY	105	
C. TYPE:	4. AMOUNT:			78	234	
E. KIND OF AWARD:	E. CUM. AMT.		21. PERFORMING ORGANIZATION			
NAME: <sup>h</sup> Walter Reed Army Institute of Research		NAME: <sup>h</sup> Walter Reed Army Institute of Research		PRINCIPAL INVESTIGATOR (Pencode SEAN II U.S. Academic Institution)		
ADDRESS: <sup>h</sup> Washington, DC 20012		ADDRESS: <sup>h</sup> Washington, DC 20012		NAME: <sup>h</sup> Davidson, D. E. LTC		
TELEPHONE:		TELEPHONE: 202-576-2292		SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]		
22. GENERAL USE		Foreign intelligence not considered		ASSOCIATE INVESTIGATORS		
NAME: <sup>h</sup>		NAME: <sup>h</sup>		NAME: <sup>h</sup>		
22. KEYWORDS (Pencode EACH with Security Classification Code)						
(U) Plasmodium; (U) Malaria; (U) Drug Development; (U) Antimalarials; (U) Biology; (U) Chemistry; (U) Pharmacodynamics; (U) Drug Metabolism; (U) Toxicology						
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Pencode individual paragraphs identified by number. Pencode last of each with Security Classification Code.)						
23. (U) To conduct in-house and contract studies in biology specifically related to the design, development and exploitation of new antimalarials for military use against drug resistant strains.						
24. (U) Close supervision will be maintained by providing guidance and an integrated evaluation of productivity, and by the redirection and coordination of objectives as dictated by feedback from clinical studies as candidate antimalarials.						
25. (U) 76 10 - 77 09 Compounds are tested for suppressive causal prophylactic or radical curative antimalarial activity in approximately 25 test systems at 6 different laboratories. Of 5658 compounds screened in mice in the primary P. berghei suppressive test, 734 were active. Of these, approximately 125 have been selected for advanced study, including 15 which have been tested against P. cynomolgi and/or P. falciparum in subhuman primates. A total of 796 compounds were tested for causal prophylactic activity in the rodent malaria model; 263 compounds were active via subcutaneous administration, and 88 were active orally. A total of 101 causal prophylactic agents were tested as radical curative agents against P. cynomolgi in rhesus monkeys; 39 were active; and, of these, 5 were more than 4 times as active as primaquine. A mouse model to evaluate compounds for repository antimalarial activity was designed and used to test 30 compounds. Ten compounds had repository action beyond 17 days, and three beyond 90 days. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.						
1297						

\*Available to contractors upon affirmative approval.

DD FORM 1498  
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68  
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M762770A803 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 086 Biological Evaluation of Antimalarial Drugs

Investigators.

Principal: LTC David E. Davidson, Jr., VC  
Gerald J. McCormick, Ph.D.  
MAJ Lyle L. Ketterling, MSC  
Marie M. Grenan  
Gloria P. Willet

Candidate chemical compounds are tested for prophylactic or suppressive antimalarial activity employing some 25 animal and in vitro test systems in-house and in 6 contractor laboratories.

A. Studies of malaria parasite metabolism and drug action.

1. Description:

The objectives of these studies are to define metabolic alterations of human and animal red blood cells when infected with malaria parasites, to elucidate the metabolism of the parasite, and to assess the effect of antimalarial drugs on the erythrocytic alterations, parasite metabolic activity, and the course of infection, in order to guide the development of new drugs and preparations effective against resistant falciparum malaria, a disease of continuing military importance. Studies are conducted both in vivo and in vitro.

2. Progress:

a. Studies of drug preparations implanted in vivo have continued in collaboration with Dynatech R/D Company, under the protocol entitled "Development of an implantable sustained release system for the prevention of malaria". A report of a completed study of preparations with WR 158122 was published (1). Studies of preparations with combinations of WR 158122 and sulfadiazine are in progress. Efficacy against Plasmodium berghei in mice has been found in an implanted preparation with dosages of 10 mg/kg for WR 158122 and 100 mg/kg for sulfadiazine. Efficacy of WR 158122 itself was observed over a four week period, and in a study of this drug in combination with diacetyldiaminodiphenylsulfone (DADDS), efficacy was observed in a combination with dosages of 2 mg/kg for WR 158122 and 10 mg/kg for DADDS in the two week infective challenge test.

b. Studies with P. falciparum to determine requirements of growth during cultivation in vitro have continued. Several fatty

acids have been found to contribute to maturation of parasites during cultivation, and investigations are in progress with respect to effective concentrations and combinations of these compounds (oleic, cis-vaccenic, and elaidic acids).

c. Several studies with P. knowlesi have been completed. A report of a study of levels of lipids in plasma of normal and infected rhesus monkeys, done in collaboration with the University of Maryland, was published (2). Reports of studies of metabolism in vitro which detailed the discovery of a metabolic pathway (biosynthesis of methionine) and the relationship of this pathway to thymidylic acid biosynthesis and folic acid metabolites, were published (3) and accepted for publication (4).

B. Infection of Bolivian Owl Monkeys (*Aotus trivirgatus*) with *Plasmodium falciparum* trophozoites.

1. Objective:

To determine whether *Aotus trivirgatus* of Bolivian origin will support infection with trophozoite-induced *Plasmodium falciparum* malaria.

2. Background:

Laboratory infections have been successfully induced by intravenous administration of trophozoites of P. falciparum to Owl monkeys of Panamanian (1) or of Columbian origin (2, 3, 4, 5), but neither of these monkeys can be procured in the United States in sufficient numbers to support malaria studies. Attempts to establish falciparum malaria in Peruvian Owl monkeys have failed (3). The inability of various sub-species of Owl monkeys to support falciparum malarias may well be genetically determined, although cross-immunity from intercurrent filarial infection has also been implicated (3).

3. Progress:

The poly-drug resistant Smith strain of Plasmodium falciparum, which had previously been adapted to the Colombian *Aotus* monkey by Schmidt (3) was successfully transferred to splenectomized Bolivian *Aotus* monkeys and was subsequently carried through 4 blood passages in non-splenectomized Bolivian *Aotus*.

All of the Bolivian *Aotus* were of karyotype VI of Ma and Jones (49/50), and with a single exception, they were free of blood parasites prior to use. One monkey (#A137) had circulating micro-filaria of Dipetalonema sp. The infection was passed by intravenous administration of freshly collected, heparinized whole blood

containing  $10^8$  parasitized erythrocytes. The original donor monkey was a Colombian Aotus.

The results of 5 passages of Smith Strain P. falciparum in 7 Bolivian Aotus monkeys are presented in Table 1:

Table 1  
Passage of Smith Strain Plasmodium falciparum in  
Bolivian Aotus Monkeys

<u>Passage #</u>	<u>Monkey #</u>	<u>Days to Patency</u>	<u>Peak Parasitemia % (DAY)</u>	<u>Death</u>
1	A-143 (splenectomized)	3	34% (18)	Day 24
	A-144 (splenectomized)	3	19% (7)	Day 11
2	A-148 (splenectomized)	<5	18% (16)	Day 19
	A-145 (intact)	<5	5% (7)	Survived
3	A-138 (intact)	4	18% (14)	Day 15
4	A-137 (intact-micro-filaremia)	14	11% (22)	Day 22
5	A-139 (intact)	7	3% (9)	Survived

In splenectomized monkeys, the onset of parasitemia was prompt (day 3-5); parasitemia increased progressively, reaching levels of 18-34%, producing fatality in all 3 monkeys in 11-24 days.

In intact monkeys, the onset of parasitemia was slightly delayed, and the peak parasitemia was less severe. Two of four intact monkeys developed parasitemia of only 3-5%, and thereafter parasitemia became subpatent through a 60 day follow-up period. The other two intact monkeys terminated fatally. Notably, one monkey (# D137), which was naturally infected with microfilaria of Dipetalonema, exhibited a much prolonged prepatent period (14 days).

#### 4. Conclusion:

It appears that Aotus monkeys of Bolivian origin may be a suitable experimental host for falciparum malaria.

#### C. Phototoxicity Testing of Antimalarial Compounds in Mice.

##### 1. Description:

Certain quinolinemethanol antimalarial drugs (e.g., SN 10275) induce a phototoxic reaction in experimental animals and in man. This side effect has been overcome by structural modification,

and 2 non-phototoxic quinolinemethanol antimalarials (WR 30090 and mefloquine) have been tested successfully in man. In connection with further efforts to develop more effective quinolinemethanols, phenanthrenemethanols, pyridinemethanols and related antimalarial compounds for human use, these compounds are tested for phototoxic side effects in mice using methods described in previous annual reports. For these studies ICR albino mice are exposed to ultraviolet light through window glass for 70 hours at a distance of 30 cm from two F40-BLB lamps (dose rate  $4.9 \times 10^3$  ergs/cm<sup>2</sup>/sec; wavelength range 3200-4000 Å). Phototoxicity is scored based upon daily observation of skin changes over a 10-day post-exposure period. The experimental drugs are administered 1 hour before the UV exposure is initiated.

2. Progress:

Four quinolinemethanols were tested. Two were not phototoxic (WR 226663 and WR 228974). One (WR 53188) was slightly phototoxic at high dose levels (320 mg/kg); and one (WR 228974) was highly phototoxic (<20 mg/kg).

A series of naphthalenemethanols which are structural analogues of the quinolinemethanol series have been developed. Several of these are potent antimalarials. Five of these naphthalene-methanols have been tested for phototoxicity. Three were not phototoxic (WR 181613, WR 183756, and WR 230331), although their exact quinolinemethanol counterparts were phototoxic. The other two naphthalenemethanols (WR 208660 and WR 228340) were only slightly phototoxic at high doses (160 mg/kg).

Six phenanthrenemethanols were tested. Four (WR 100351, WR 126927, WR 131834, WR 146419) were not phototoxic, One (WR 173872) was slightly phototoxic (160 mg/kg); and one (WR 171669) was moderately phototoxic. WR 171669 was considerably more phototoxic by the oral route (40 mg/kg) than by the subcutaneous route (80 mg/kg).

D. Screening of Primaquine Analogues for Potential Hemolytic Side Effects.

1. Description:

Primaquine and related 8-aminoquinoline antimalarial drugs induce hemolysis in individuals whose erythrocytes are deficient in glucose-6-phosphate dehydrogenase (G-6-PD). This serious side-effect constitutes the greatest hazard from the use of primaquine among males of populations in which this sex-linked genetic defect is prevalent. G-6-PD deficiency is common among Mediterranean and Oriental populations, and occurs in approximately 10% of American black males.

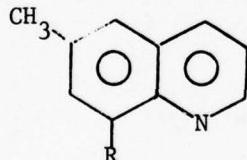
An in vitro test has been designed to screen 8-amino-quinolines and other experimental antimalarial drugs for their hemolytic potential, based upon the hypothesis that the hemolytic activity of primaquine is related to its stimulation of the hexose monophosphate shunt (HMS), which, in G-6-PD deficient cells, leads to hemolysis through accumulation of peroxides.

The in vitro test utilizes freshly collected normal human erythrocytes and serum which are incubated for 90 minutes at 37°C in phosphate-buffered saline, pH 7.2, in the presence of 0.5  $\mu$ Ci of glucose-1- $^{14}\text{C}$  and the test drug at  $2.5 \times 10^{-3}\text{M}$ . The evolution of  $^{14}\text{CO}_2$  above baseline values is indicative of increased activity of the HMS. For each test drug, a primaquine index is calculated by comparing the amount of increase in  $^{14}\text{CO}_2$  production in the test drug tube with the amount of increase in  $^{14}\text{CO}_2$  production in the primaquine control tube. Primaquine indices greater than 1 are indicative of potential hemolytic activity greater than that of primaquine.

2. Progress:

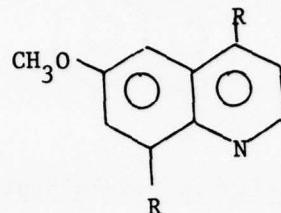
Approximately 100 antimalarial compounds of the 8-amino-quinoline class were tested in vitro to determine their ability to stimulate glucose metabolism through the hexose monophosphate shunt in human erythrocytes. Some of these data are presented in Tables 1 - 3.

Table 1:  
Potential Hemolytic Activity of Side Chain Variants of Primaquine



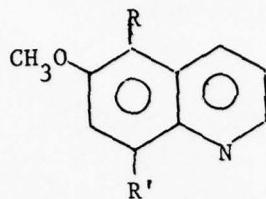
<u>WR #</u>	<u>Side Chain (R)</u>	<u>Primaquine Index</u>
2975	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> NH <sub>2</sub> (Primaquine)	1.0 (by definition)
152149	-NH(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> )NH <sub>2</sub> (Quinocide)	1.24
4234	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> N(C <sub>2</sub> H <sub>5</sub> ) (Pamaquine)	1.98
6012	-NH(CH <sub>2</sub> ) <sub>5</sub> NHCH(CH <sub>3</sub> ) <sub>2</sub> (Pentaquine)	1.52
6020	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> NHCH(CH <sub>3</sub> ) <sub>2</sub> (Isopentaquine)	0.77

Table 2


Potential Hemolytic Activity of 4-Substituted 8-Aminoquinolines

<u>WR</u>	<u>4-Substituent (R)</u>	<u>Side Chain (R')</u>	<u>Primaquine Index</u>
181023	-CH <sub>3</sub>	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> NH <sub>2</sub>	0.34
208442	-CH <sub>2</sub> CH <sub>3</sub>	"	0.16
218806	-CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	"	0.64
218805	-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	"	0.51
211663	-CH = CH <sub>2</sub>	"	0.27
215300	-CH <sub>2</sub> OH	"	1.44
216837	-CF <sub>3</sub>	"	0.23
217271	-OCH <sub>3</sub>	"	0.23
214198	-OH	"	0.30
212293	-NH <sub>2</sub>	"	0.56
214703	-SCH <sub>3</sub>	"	0.72
215296	-CH <sub>3</sub>	-NH(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> )NH <sub>2</sub>	0.11
212624	-CH <sub>3</sub>	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>4</sub> NH <sub>2</sub>	0.37
212579	-CH <sub>3</sub>	-NH(CH <sub>2</sub> ) <sub>4</sub> CH(CH <sub>3</sub> )NH <sub>2</sub>	0.95
215761	-CH <sub>3</sub>	-NHCH(C <sub>2</sub> H <sub>5</sub> )(CH <sub>2</sub> ) <sub>3</sub> NH <sub>2</sub>	0.40
226573	-CH <sub>3</sub>	-NHCH(C <sub>3</sub> H <sub>7</sub> )(CH <sub>2</sub> ) <sub>3</sub> NH <sub>2</sub>	0.75
218335	-CH <sub>3</sub>	-NH(CH <sub>2</sub> ) <sub>3</sub> CH(C <sub>2</sub> H <sub>5</sub> )NH <sub>2</sub>	0.12
211816	-CH <sub>3</sub>	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> N(C <sub>2</sub> H <sub>5</sub> )	0.52
127854	-CH <sub>3</sub>	-NH(CH <sub>2</sub> ) <sub>5</sub> NHCH(CH <sub>3</sub> ) <sub>2</sub>	0.54
6027	-CH <sub>3</sub>	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> NHCH(CH <sub>3</sub> ) <sub>2</sub>	1.20

Table 3

Potential Hemolytic Activity of 5-Substituted 8-Aminoquinolines

<u>WR</u>	<u>(R)</u> <u>5-Substituent</u>	<u>Other Substit.</u>	<u>Side Chain (R')</u>	<u>Primaquine Index</u>
5990	-OCH <sub>3</sub>	-	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> NH <sub>2</sub>	6.92
218676	-OCH <sub>2</sub> CH <sub>3</sub>	-	"	3.53
228583	-OCH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	-	"	1.75
228000	-O(CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub>	-	"	1.28
228708	-O(CH <sub>2</sub> ) <sub>5</sub> CH <sub>3</sub>	-	"	0.38
6877	-OCH <sub>3</sub>	-	-NH(CH <sub>2</sub> ) <sub>5</sub> NHCH(CH <sub>3</sub> ) <sub>2</sub>	3.97
226937	-OCH <sub>3</sub>	-	-NH(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> )NH <sub>2</sub>	1.48
210448	-OCH <sub>3</sub>	1-CH <sub>3</sub>	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> NH <sub>2</sub>	2.06
216804	-OCH <sub>3</sub>	4-CH <sub>3</sub>	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> NH <sub>2</sub>	2.76
221527	-OCH <sub>3</sub>	4-CH <sub>3</sub>	-NH(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> )NH <sub>2</sub>	1.72
228710	-OCH <sub>3</sub>	4-CH <sub>3</sub>	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>4</sub> NH <sub>2</sub>	3.14
226899	-OCH <sub>3</sub>	4-CH <sub>3</sub>	-NH(CH <sub>2</sub> ) <sub>4</sub> CH(CH <sub>3</sub> )NH <sub>2</sub>	2.02
226426	-OCH <sub>3</sub>	4-CH <sub>3</sub>	-NHCH(C <sub>2</sub> H <sub>5</sub> )(CH <sub>2</sub> ) <sub>3</sub> NH <sub>2</sub>	2.18
219423	-OCH <sub>3</sub>	4-CH <sub>3</sub>	-NH(CH <sub>2</sub> ) <sub>5</sub> NHCH(CH <sub>3</sub> ) <sub>2</sub>	2.52
228002	-OCH <sub>3</sub>	2,4-CH <sub>3</sub>	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> NH <sub>2</sub>	2.90
228456	-OCH <sub>3</sub>	2,4-CH <sub>3</sub>	-NH(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> )NH <sub>2</sub>	2.45
199507	-OH	-	-NHCH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> NH <sub>2</sub>	8.10
194333	-Cl	-	"	0.48
218681	-Cl	4-CH <sub>3</sub>	"	1.02
200073	-Br	-	"	0.97
215733	-F	2-CH <sub>3</sub>	"	3.80
219874	-F	4-CH <sub>3</sub>	"	6.77
210810	-CH <sub>3</sub>	2-CH <sub>3</sub>	"	1.01
221041	-SCH <sub>3</sub>	-	"	0.77
224640	-N(CH <sub>3</sub> ) <sub>2</sub>	-	"	4.60

Side-chain variations (Table 1) influenced the potential hemolytic activity. Isopentaquine (WR 6020) had slightly less activity than primaquine (PI=0.77). The other examples (quinocide, pentaquine, and pamaquine were more active, with primaquine indices of 1.24-1.98.

The addition of substituents in the 4-position of the quinoline ring (Table 2) generally reduced hemolytic potential, regardless of the nature of the substituent. Substitution in the 5-position (Table 3) dramatically enhanced hemolytic potential; 5-methoxy, 5-hydroxyl, 5-fluoro, and 5-dimethylamine analogues were 4 to 8 times as active as primaquine, while 5-chloro, 5-bromo and 5-thiomethyl substituents did not enhance hemolytic potential. Hemolytic potential progressively decreased as the length of a 5-alkyloxy substituent was increased from 1 to 6 carbons.

Project 3M762770A803 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 086 Biological Evaluation of Antimalarial Drugs

Literature Cited.

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4. Smith, C. C., McCormick, G. J., and Canfield, C. J.: Plasmodium knowlesi: In vitro Biosynthesis of Methionine and Thymidylic Acid. Experimental Parasitology, in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>b</sup>	2. DATE OF SUMMARY <sup>b</sup>	REPORT CONTROL SYMBOL	
3. DATE PREV SURY	4. KIND OF SUMMARY	5. SUMMARY SCTY <sup>b</sup>	6. WORK SECURITY <sup>b</sup>	7. REGADING <sup>b</sup>	8. DESIGN INSTN <sup>b</sup>	9. SPECIFIC DATA-CONTRACTOR ACCESS	
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES <sup>b</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		10. LEVEL OF SUM	
B. PRIMARY	62770A	3M762770A803		00		A. WORK UNIT WORK UNIT NUMBER	
C. CONTRIBUTING							
C. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) <b>(U) Determination of Pharmacological Effects of Antimalarial Drugs</b>							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS 012600 Pharmacology 002600 Biology							
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD			
72 07	CONT	DA		C.	In-house		
17. CONTRACT/GANT		18. RESOURCES ESTIMATE					
19. DATES/EFFECTIVE: NA EXPIRATION:		FISCAL	20. PROFESSIONAL MAN YRS	21. FUNDS (in thousands)			
20. NUMBER: <sup>b</sup>		YEAR	77	9.0	390		
21. TYPE:		CURRENT	78	9.0	365		
22. KIND OF AWARD:		E. CUM. AMT.	23. PERFORMING ORGANIZATION				
24. RESPONSIBLE DOD ORGANIZATION		NAME: Walter Reed Army Institute of Research					
NAME: Washington, DC 20012		Div of Medicinal Chemistry					
ADDRESS: <sup>b</sup>		ADDRESS: <sup>b</sup> Washington, DC 20012					
RESPONSIBLE INDIVIDUAL		PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)					
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25. GENERAL USE		SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]					
Foreign intelligence not considered		ASSOCIATE INVESTIGATORS					
		NAME: CHUNG, Dr. H. NAME: ROZMAN, Dr. R. S.					
26. KEYWORDS (Precede each with Security Classification Code) (U) Pharmacodynamics; (U) Pharmacokinetics; (U) Toxicity; (U) Biotransformation; (U) Antimalarial Drugs; (U) Preclinical Pharmacology							
27. TECHNICAL OBJECTIVE <sup>b</sup> ; 28. APPROACH, 29. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) The technical objectives are to develop and exploit animal models, for the study of the pharmacodynamic and toxic effects of drugs intended for use as antimalarials in man. The intended purposes of these studies are to provide a basis for predicting human response and to fulfill requirements for submission of IND for clinical trials of new antimalarials for military personnel in malarious areas.							
24. (U) The approach is to study both the effects of antimalarial drugs on healthy animals and the fate of these drugs in healthy animals in order to predict the human tolerance to new drugs (Phase I). The handling of antimalarial drugs by diseased animals is being studied to determine the effects of malaria upon pharmacokinetics. This is in order to predict the tolerance of new antimalarial drugs in human efficacy studies (Phase II).							
25. (U) 76 10 - 77 09 Technical management continued for 12 contracts in pharmacology. Four new IND applications and 5 supplements were written. Absorption, distribution and excretion studies using radioactively labeled WR 172,435 and WR 177,602 were carried out in mice. Cardiorespiratory evaluations in anesthetized dogs were made on WR 142,490, WR 172,435, WR 177,602 and WR 194,965. Methods for determining blood levels of 4 antimalarials utilizing extraction and high pressure liquid chromatography with a versatile mobile phase were developed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.							
1307							
<small>*Available to contractors upon originator's approval.</small>							
DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE							

Project 3M762770A803 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 087 Determination of pharmacological effects of antimalarial drugs

Investigators.

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1. Description.

The thrust of the pharmacological investigations carried out by the department continues in two broad areas. First is the effect of the body or system on the drug, i.e., absorption, distribution, bio-transformation and excretion. Second is the effect of the drug on the body or system. A considerable overlap exists between the two areas. This year the emphasis placed on developing sensitive assay methods for several of the new antimalarial drugs in biological fluids also continued.

2. The absorption, distribution and excretion of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H in mice.

a. Background:

The compound 3-di-n-butylamino-1-[2,6-bis(4-fluoromethyl-phenyl)-4-pyridyl]-propano<sup>T</sup> methanesulfonate (WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H) is the second pyridinemethanol that is a promising candidate anti-malarial drug. The objective of this study was to determine the absorption, distribution and excretion of this compound in mice.

b. Materials and methods:

Radiolabelled WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H (17.8091  $\mu$ Ci/mg; Lot No. 496-3a) was synthesized by Monsanto Research Corp. (Dayton, OH) with the <sup>14</sup>C-label in the methanol carbon. The nonradiolabelled compound (Lot AK; bottle no. BE32210) was prepared by Ash Stevens, Inc. (Detroit, MI). A suspension of WR 172,435-<sup>14</sup>C·CH<sub>3</sub>SO<sub>3</sub>H with a specific activity of 3  $\mu$ Ci/mg was prepared in 0.2% methylcellulose and 0.4% Tween 80 in deionized water. Chemical and radiochemical purity of the compound were assayed by thin-layer chromatography.

A premixed liquid scintillation solution, Hydromix (Yorktown Research Co., S. Hackensack, NJ) was used for radioassay. All other chemicals and solvents used were reagent grade quality.

Albino, ICR male mice from the Walter Reed Colony, weighing about 25 g, were used. The mice were fed D and G Laboratory Diet (G.L. Baking Co., Frederick, MD), and were maintained in a temperature controlled room with a 12-hr light-dark cycle. The mice were fasted for about 18 hr prior to dosing but were permitted water ad libitum. A dose of 20 mg/kg was administered to each mouse by oral intubation. The mice were then housed 4 each in 7 modified Roth metabolism cages and were allowed water ad libitum. Animals remained fasted for 4 hr after dosing. Standard doses were taken for radioassay before, during and after dosing the animals.

Complete urine samples were collected from 2 cages every 12 hr for 5 days and every 24 hr for 3 days thereafter. Cages were rinsed at each collection time with deionized water and the washings added to the urine samples. Aliquots of each sample were taken for total radioactivity determination. The samples were lyophilized to dryness and then extracted with absolute methanol. Extracts were measured and aliquots taken for total radioactivity determination. The extracts were evaporated to dryness in a flash evaporator at temperatures less than 40°C, reconstituted with small amounts of absolute methanol, and stored in a freezer at -10°C for subsequent TLC study.

Complete fecal samples were collected from the same cages at the same time intervals as the urine samples (the cages were rinsed at each collection time with methanol and the washings added to the fecal samples), homogenized in absolute methanol in a Waring blender and extracted in glass columns with absolute methanol. The eluates were measured and aliquots taken for total radioactivity determination. The methanol extracts were evaporated to dryness in a flash evaporator at temperatures less than 40°C, reconstituted in small amounts of absolute methanol, aliquots taken for radioactivity determination, and the samples stored as described above.

Plasma and red blood cell (RBC) levels were examined. At appropriate intervals after dosing, the 4 mice in a group were anesthetized with ether and exsanguinated via a surgically exposed femoral artery. Aliquots of the heparinized pooled blood were used for radioassay along with aliquots for the hematocrit (Hct) determination (13,000 x g for 3 min). The remainder of each sample was centrifuged at 7,000 x g for 5 min to separate the plasma from the red blood cells. Aliquots of plasma were taken from each sample for radioassay. Red blood cell levels were calculated. The plasma was lyophilized and extracted with methanol.

Tissue levels of radioactivity were determined at appropriate intervals after dosing. All 4 mice in a group were anesthetized with ether, exsanguinated by cutting a femoral artery and the following tissues removed by dissection: submaxillary salivary glands, heart, lungs, liver, kidneys, spleen, gall bladder and bile, gastrointestinal tract and contents, eyes and brain. Samples of abdominal fat and skeletal muscle were also taken. Each tissue type from each time interval group was pooled, placed in a preweighed container, weighed and sufficient methanol was added to cover the tissues. The tissues and carcasses were stored as described above until later processing.

Each tissue pool, as well as the carcasses, was homogenized at room temperature with methanol in a Waring blender. Aliquots were taken from each homogenate for radioassay and the remaining homogenate was packed into a glass column. After the homogenate was thoroughly extracted with methanol, the volumes of the methanol eluates were measured and aliquots were taken for radioassay. The remainder of each eluate was evaporated to dryness and reconstituted in small amounts of methanol. Again samples were taken for radioassay and the remainders were stored as described above for subsequent TLC analysis.

For radioassay, 15 ml of premixed scintillation fluid, Hydromix, was added to each sample. The aliquots were counted for 10 min in a Searle Mark II liquid scintillation counter. Quenching and counting efficiency were corrected by external standardization.

Thin layer chromatography (TLC) was used extensively for isolation of the drug. Appropriate amounts of samples were streaked on EM pre-coated silica gel F-254 TLC plates (EM Laboratories, Ltd., Elmsford, NY) and developed for 10 cm from the origin using n-butanol:acetic acid:water (10:1:1 by volume) as the solvent system. After air drying, the plates were visualized with UV light (254 nm) and scanned with a Varian Model 6000 Radioscanner with integrator (time constant = 1 sec; speed = 4 in/hr; attenuation = 10 cps). A standard streak of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H was developed on each plate as a comparison standard.

c. Results and discussion:

WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H was studied over a 192 hr period after a single oral dose of 20 mg/kg of the <sup>14</sup>C-labelled drug was administered to mice. About 76% of the administered radioactivity was excreted in feces and only 0.72% of the radioactivity was excreted in urine during this time period (Table 1). The final total percent <sup>14</sup>C recovery, including residual carcasses (18.82%), was 95.94%.

Radioactivity in feces was analyzed by TLC after development in n-butanol:acetic acid:water (10:1:1 by volume). Two major peaks

were detected and one of these peaks was found to be identical to the parent drug. The percent of parent drug in each sample is shown in Table 2. Up to 60 hr after dosing, the percent of parent drug in the fecal samples ranged from 36.4% to 48.6%. This indicates rapid metabolism of the drug. Only one major metabolite was detected.

The concentrations of  $^{14}\text{C}$ -drug equivalents in the plasma and RBCs of the mouse were determined after a single oral dose of the drug. The plasma and RBC concentration curves for total  $^{14}\text{C}$ -drug equivalents, expressed as  $\mu\text{g}$  of drug equivalents per ml, are summarized in Table 3. The elimination half-lives ( $T_{1/2}$ ) of plasma and RBC radioactivity were calculated by linear regression analysis of the concentration vs. time plots to be 1.95 hr and 4.02 hr respectively.

The distribution of WR 172,435- $^{14}\text{C}$  in selected tissues was studied. Of the organs investigated, the major sites of distribution of WR 172,435-derived radioactivity were the liver, gall bladder plus bile, kidneys, submaxillary salivary glands, lungs and spleen 2 hr after oral administration of the drug (Table 4). Two hr after the administration of the drug, a total of about 25% of the dose was accounted for in the selected tissues plus residual carcasses, excluding the G.I. tract and its contents. One-hundred and twenty hr after the oral administration of the drug, the major sites of deposition of drug-derived radioactivity were the liver, kidneys, lungs, submaxillary salivary glands, spleen, eyes and abdominal fat.

### 3. The absorption, distribution and excretion of WR 177,602-HCl in mice.

#### a. Background:

Threo- $\alpha$ -(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinoline-methanol hydrochloride (WR 177,602-HCl) is a diastereoisomer of the important new antimalarial WR 142,490-HCl. The objective of this study was to determine the absorption, distribution and excretion of this compound in mice.

#### b. Materials and methods:

Radiolabelled WR 177,602-HCl (32.3427  $\mu\text{Ci}/\text{mg}$ ; Lot No. 469a-3-1) was synthesized by Dr. W. H. Yanko of Monsanto Research Corp. (Dayton Laboratory, Dayton, OH) with the  $^{14}\text{C}$ -label on the methanol carbon. The nonradiolabelled compound (Lot AD; bottle number BE 77728) was prepared by Cordova Chemical Co. (Sacramento, CA). A suspension of WR 177,602- $^{14}\text{C}$ -HCl with a specific activity of 1.62  $\mu\text{Ci}/\text{mg}$  was prepared in 0.2% methylcellulose and 0.4% Tween 80 in deionized water. Chemical and radiochemical purity of the compound were assayed by thin-layer chromatography. A premixed liquid scintillation solution,

Hydromix (Yorktown Research Co., S. Hackensack, NJ), was used for radioassay. All other chemicals and solvents used were reagent grade quality.

Eight albino, ICR female mice from the Walter Reed Colony weighing about 25 g were used. The mice were fed D and G Laboratory Diet (G. L. Baking Co., Frederick, MD), and were maintained in a temperature controlled room with a 12-hr light-dark cycle. The mice were fasted for about 18 hr prior to dosing but were permitted water ad libitum. A dose of 10 mg/kg was administered to each mouse by oral intubation. The mice were then housed 4 each in 2 modified Roth metabolism cages and were allowed water ad libitum. Animals remained fasted for 4 hr after dosing. Standard doses were taken for radioassay before, during and after dosing the animals.

Complete urine samples were collected every 12 hr for 3 days and every 24 hr for 4 days thereafter (cages were rinsed at each collection time with deionized water and the washings added to the urine samples). Aliquots of each sample were taken for total radioactivity determination. The samples were lyophilized to dryness and then extracted in glass columns with absolute methanol. Eluates were measured and aliquots taken for total radioactivity determination. The extracts were evaporated to dryness in a flash evaporator at temperatures less than 40°C, reconstituted with small amounts of absolute methanol and aliquots taken for radioassay. The samples were stored at -10°C for later TLC study.

Complete fecal samples were collected at the same time intervals as the urine samples (the cages were rinsed at each collection time with methanol and the washings added to the fecal samples), homogenized in absolute methanol in a Waring blender and extracted as above. The eluates were measured and aliquots taken for total radioactivity determination. The methanol extracts were evaporated to dryness in a flash evaporator at temperatures less than 40°C, reconstituted in small amounts of absolute methanol and aliquots taken for radioassay. The samples were stored as described above.

At 168 hr, each of the 4 mice in a group were anesthetized with ether, exsanguinated by unilateral transection of the femoral vessels and the following tissues removed by dissection: submaxillary salivary glands; heart; lungs; liver; kidneys; spleen; gall bladder and bile; gastrointestinal tract and contents; eyes and harderian glands; and brain. Samples of abdominal fat and skeletal muscle were also taken and the residual carcasses stored in methanol at -10°C for later processing. Each tissue type from each of the 2 groups was pooled separately, placed in a preweighed

container, weighed and sufficient methanol was added to cover the tissues. The tissues were stored at -10°C until the following processing could be carried out.

Each tissue pool was homogenized at room temperature with methanol in a Waring blender. Aliquots were taken from each homogenate for radioassay and the remaining homogenate was packed into a glass column. After the homogenate was eluted and the eluate passed once through the solids, 3 to 4 bed volumes of additional methanol were passed through slowly. The volume of the total methanol eluate of each pool was measured and aliquots were taken for total radioactivity determination. The remainder of each eluate was evaporated to dryness as described above, reconstituted in small amounts of methanol and aliquots taken for radioassay. The samples were stored at -10°C for subsequent TLC analysis.

The animal carcasses were homogenized in methanol using a Tekmar tissue grinder. The homogenates of the carcasses were processed as described for tissue homogenates.

Fifteen ml of premixed scintillation fluid, Hydromix, were added to each aliquot. The samples were counted for 10 min in a Searle Mark II liquid scintillation counter. Quenching and counting efficiency were corrected by external standardization to obtain the dpm.

c. Results and discussion:

The disposition of threo- $\alpha$ -(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride (WR 177,602-HCl) was studied over a 168 hr period after a single oral dose of 10 mg/kg of the  $^{14}\text{C}$  labelled drug was administered to mice. By about 168 hr, 70% of the administered radioactivity was excreted in the feces, 23% of the radioactivity was excreted in the urine, 0.56% of the radioactivity was detected in the selected tissues, and 1.54% of the radioactivity was found in the residual carcasses. Approximately 95.2% of the administered dose was recovered (Table 5). The excretion of the drug-derived radioactivity in the urine and feces peaked between 24 and 36 hr.

The elimination half-lives ( $T_{\frac{1}{2}}$ ) of urine and fecal radioactivity were 13.9 hr and 13.8 hr respectively, as calculated by linear regression analysis of the "percent remaining radioactivity versus time" plot.

The distribution of radioactivity in selected tissues at 168 hr was determined. Of the organs investigated, the major sites of concentration of WR 177,602-derived radioactivity, expressed as

drug-equivalents, were the gall bladder and bile (145.93 µg/g) and adrenal glands (9.02 µg/g) (Table 6). The greatest percentage of the administered dose was found in the liver and gastrointestinal tract plus contents (Table 6).

4. A comparison of the cardiovascular and respiratory effects of WR 177,602·HCl and WR 142,490·HCl in the anesthetized beagle dog.

a. Background:

This investigation was undertaken to describe the acute cardiovascular and respiratory effects of the candidate antimalarial drug threo- $\alpha$ -(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride (WR 177,602·HCl) after intravenous injection to anesthetized beagles. Since WR 177,602·HCl is the threo isomer of the new antimalarial drug mefloquine HCl (WR 142,490·HCl), the cardiovascular and respiratory effects of mefloquine were also assessed in order to compare the relative potencies of the 2 compounds in an identical screening procedure.

b. Materials and methods:

Twenty-one male beagles, weighing from 8.6 to 14.1 kg, were anesthetized with 35 mg/kg sodium pentobarbital administered intravenously. Supplemental anesthesia was administered as needed. The left femoral artery and vein were catheterized for measurement of arterial pressure and drug administration respectively. An endotracheal tube was positioned and respiration was monitored with a pneumotachometer. The Lead II electrocardiogram was recorded and its signal was fed into a cardiotachograph for determination of heart rate. All measurements were recorded on a Hewlett-Packard 7758A series recorder. Body temperature was monitored via a rectal thermistor probe and maintained near normal by a heating pad. The hematocrit was obtained from venous blood collected by capillary tube and centrifuged for 5 min in a Clay Adams Autocrit centrifuge. At the conclusion of the experiment, animals still alive were euthanized with an overdose of sodium pentobarbital.

WR 177,602·HCl, Lot AE, was obtained from bottle number BG 58518. WR 142,490·HCl, Lot AH, was obtained from bottle number BE 16387. Since WR 177,602·HCl and WR 142,490·HCl have identical molecular weights, equal dosages based on the weight of the salt of the two compounds were used. The drug vehicle for both compounds was a 1:1:3 solution of ethanol:propylene glycol:isotonic saline.

The animals were divided into 4 groups. The first group of 6 beagles was used to determine the cumulative effect of increasing doses of WR 177,602·HCl (3 beagles) and WR 142,490·HCl (3 beagles)

on the cardiovascular and respiratory systems. The drug concentration for this study was 50 mg/ml and the solutions were maintained at 50-60°C between injections to prevent precipitation. The appropriate drug was administered as an intravenous bolus at 20 min intervals in increasing doses and the cardiovascular and respiratory responses were monitored. Progressive doses of 1 mg/kg, 1 mg/kg, 3 mg/kg, 3 mg/kg, 10 mg/kg ... were administered until death occurred. The cumulative fatal dose was recorded. Two analyses of the data were made. The first was a comparison of the baseline readings obtained prior to administration of a particular dose and the maximum post-injection response. The paired *t* test of significance was used in this analysis and a computed *p* value of less than 5% was considered significant. The second analysis was of the control baseline (baseline value for the 1.0 mg/kg dose) and the succeeding baseline values. This analysis was made using Dunnett's test of significance (Zar, 1974) and a computed *p* value of less than 5% was considered significant.

A second group of 6 beagles was used to evaluate the effects of a 60 min infusion of WR 177,602-HCl (3 beagles) and WR 142,490-HCl (3 beagles). A total dose of 35 mg/kg salt of WR 177,602-HCl or WR 142,490-HCl was infused during this period. The rate of drug administration was (0.583 mg/kg)/min. The infusion rate was 0.97 ml/min so that the drug concentration was (0.583 mg/kg)/0.97 ml for a particular experiment. The animals were observed for a 2 hr period following infusion to determine if recovery to baseline values occurred. The responses at 20, 40 and 60 min after drug infusion and 30, 60, 90 and 120 min after termination of infusion were compared with baseline values using Dunnett's test of significance (Zar, 1974) with a computed *p* value of less than 5% considered significant.

A third group of 3 beagles received a one hr infusion of the drug vehicle (0.97 ml/min) followed by a 2-hr observation period. These animals were then given progressively larger injections of the drug vehicle at 20 min intervals as in the cumulative toxicity studies. The effects of the drug vehicle as an infusion or as injections were assessed in the same manner as were the candidate antimalarials described above.

The fourth group of 6 beagles was used to assess the ability of WR 177,602-HCl (3 beagles) and WR 142,490-HCl (3 beagles) to modify the cardiovascular and respiratory effects of a series of vasoactive compounds. Phenylephrine hydrochloride (3 and 10 µg/kg), isoproterenol hydrochloride (0.5 and 1.0 µg/kg), angiotensin amide (0.5 and 1.0 µg/kg), acetylcholine hydrochloride (1.0 and 2.0 µg/kg), serotonin creatinine sulfate (10.0 and 25.0 µg/kg), and histamine phosphate (1.0 and 2.0 µg/kg), administered as their salts, were injected intravenously as a bolus at 10 min intervals and the maximum

post-injection responses were recorded. A 20 min infusion of 35 mg/kg (1.75 mg/kg/min) of WR 142,490·HCl or WR 177,602·HCl was administered after the control responses to the vasoactive compounds were obtained. WR 177,602·HCl was infused at a rate of 0.8 ml/min for a drug concentration of (1.75 mg/kg)/0.8 ml while the less soluble WR 142,490·HCl was infused at 1.6 ml/min for a drug concentration of (1.75 mg/kg)/1.6 ml. The series of vasoactive compounds was again administered and the maximum post-injection responses were again recorded. The maximum post-injection responses of the vasoactive compounds were compared with their pre-injection values by the paired *t* test of significance with a computed *p* value of less than 5% considered significant. The maximum post-injection response was recorded as a percentage of the baseline value for both the control series and the post-antimalarial series. The control and post-antimalarial responses of the vasoactive compounds were then compared using Student's *t* test with a computed *p* value of less than 5% considered significant.

c. Results and discussion:

The heart rate, mean arterial pressure and respiratory responses to repeated administration of increasing doses of WR 177,602·HCl, WR 142,490·HCl and the drug vehicle are given in Tables 7-9, respectively. The drug vehicle did not produce a significant effect on these measurements. Both WR 177,602·HCl and WR 142,490·HCl produced a transient hypotension at all doses (Table 7). However, the blood pressures recovered to baseline values between injections except for 2 beagles that received 30 mg/kg of WR 177,602·HCl. These 2 animals exhibited a significant hypotension with respect to the control baseline after a cumulative dose of 28 mg/kg.

Both WR 177,602·HCl and WR 142,490·HCl had little effect on the heart rate at a dose of 1 mg/kg (Table 8). WR 177,602·HCl, 3 mg/kg, produced a significant increase in heart rate. This appears to be a reflexive tachycardia to the hypotension produced by WR 177,602·HCl. The baseline heart rate for 10 mg/kg WR 177,602·HCl was significantly less than the control baseline indicating that the tachycardia observed at 3 mg/kg was transient and the cumulative effect of WR 177,602·HCl was a negative chronotropic. This negative chronotropic action of WR 177,602·HCl overrides the reflex tachycardia when 10 mg/kg is administered. WR 142,490·HCl, 10 mg/kg, produced similar actions on the heart rate.

Both WR 177,602·HCl and WR 142,490·HCl produced significant increases in respiratory rate typified by a rapid shallow breathing (Table 9). This response was observed after 3 mg/kg of WR 142,490·HCl but the respiratory rate had returned to control levels after

20 min. The increased respiratory response to WR 177,602·HCl, 3 mg/kg, was slightly different from that of WR 142,490·HCl as the respiratory rate did not increase rapidly but had significantly increased during the 20 min interval before the 10 mg/kg injection. Injection of WR 142,490·HCl and WR 177,602·HCl, 10 mg/kg, produced significant increases in respiratory rate. This dose was fatal to the 3 beagles given WR 142,490·HCl. A second 10 mg/kg dosing of WR 177,602·HCl was fatal to one beagle but the 2 remaining beagles did not expire until they received one 30 mg/kg dose of WR 177,602·HCl.

The average cumulative lethal dose of WR 142,490·HCl was 18 mg/kg and of WR 177,602·HCl was 48 mg/kg. The major terminal event after administration of lethal doses of either WR 177,602·HCl or WR 142,490·HCl was an increasingly rapid, shallower respiration that eventually ceased altogether. Necropsy reports indicated that the lungs were congested and filled with a reddish froth, observations consistent with pulmonary edema being the cause of death in these animals. WR 142,490·HCl produced this toxicity at a significantly lower dose than did WR 177,602·HCl.

WR 177,602·HCl and WR 142,490·HCl, 35 mg/kg, were infused slowly over a one hr period in anesthetized dogs to approximate the gastric absorption of an orally administered dose. WR 142,490·HCl has been reported to be orally effective in doses of 21.9 to 43.75 mg/kg in the Aotus monkey screen (Schmidt, 1971). The heart rate, mean arterial pressure and respiratory responses to 35 mg/kg of WR 177,602·HCl or WR 142,490·HCl given over 60 min and a 0.97 ml/min infusion of the drug vehicle for 60 min are given in Tables 10-12, respectively. Neither WR 142,490·HCl, WR 177,602·HCl nor the drug vehicle infusions had any effect on respiration or mean arterial pressure. WR 142,490·HCl decreased heart rate by 29 bpm at the end of the 60 min infusion period. However, this decrease was not statistically significant. WR 177,602·HCl did not decrease the heart rate to the same extent. When the same dose of WR 142,490·HCl or WR 177,602·HCl, 35 mg/kg, was infused in 20 min instead of 60 min, both compounds produced a significant negative chronotropism (Table 13). However, WR 142,490·HCl also produced a significant hypotension and a tendency to tachypnea, again indicating a greater potency of WR 142,490·HCl.

WR 177,602·HCl, WR 142,490·HCl and drug vehicle had little effect on the venous hematocrit during the one hr infusion and 2-hr observation period (Table 14). However, there was considerable difference in the amount of hemolysis, as indicated by the reddish tint of the plasma, attributable to the various procedures. The plasma during and after the drug vehicle infusion was without observable red coloration. Two of 3 beagles given the WR 177,602·HCl

infusion had plasma with a slight reddish tint first observed after 20 min of the infusion. However, the plasma of all animals given WR 142,490·HCl had a definite reddish coloration after 20 min of the infusion. This reddish coloration became more pronounced as the infusion continued and remained throughout the observation period. It may be concluded that WR 142,490·HCl has a greater hemolytic potential than WR 177,602·HCl in this model.

The effect of a 20 min infusion of WR 177,602·HCl, 35 mg/kg, or WR 142,490·HCl, 35 mg/kg, on the heart rate, mean arterial pressure and respiratory responses of a series of vasoactive compounds are given in Tables 15-17, respectively. Neither WR 177,602·HCl nor WR 142,490·HCl altered the heart rate responses to the vasoactive compounds. WR 142,490·HCl decreased the pressor response to the alpha-adrenergic agonist, phenylephrine, as did WR 177,602·HCl, but WR 142,490·HCl also decreased the pressor response to angiotensin. Closer inspection of the angiotensin pressor response before and after WR 177,602·HCl showed that WR 177,602·HCl depressed the pressor response, although not significantly. Therefore, it appears that the decrease in pressor response observed with WR 177,602·HCl and WR 142,490·HCl was due to a nonspecific action and not alpha-adrenergic receptor blockade. WR 177,602·HCl also increased the respiratory response to isoproterenol. This may be a function of the small sample size since isoproterenol caused a decrease in respiratory rate in one of 3 animals during the control series, producing a pronounced effect on the percentage increase observed.

In conclusion, both compounds produced an immediate hypotension after bolus administration at doses ranging from 1 to 30 mg/kg. Both compounds produced a negative chronotropic and tachypnea at the higher doses. The average cumulative lethal dose was 48 mg/kg for WR 177,602·HCl and 18 mg/kg for WR 142,490·HCl. Both compounds produced pulmonary edema which was the cause of death. Infusions of 35 mg/kg of either agent over 60 min had no significant effect although a negative chronotropic tendency was observed. Both agents caused hemolysis during the infusion but that produced by WR 177,602·HCl was minimal and less severe than that produced by WR 142,490·HCl. Both WR 177,602·HCl and WR 142,490·HCl attenuated the vasopressor response to phenylephrine. However, WR 142,490·HCl also significantly decreased the pressor response to angiotensin, indicating that the decreased pressor response is due to mechanisms other than alpha-adrenergic receptor blockade. The data indicate that WR 177,602·HCl was less potent in producing changes in cardiovascular and respiratory responses than was WR 142,490·HCl.

5. The cardiovascular and respiratory effects of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H and WR 194,965·H<sub>3</sub>PO<sub>4</sub>.

a. Background:

This investigation was undertaken to describe the acute cardiovascular and respiratory effects of the candidate antimalarial drugs 3-di-n-butylamino-1-[2,6-bis(4-trifluoromethylphenyl)-4-pyridyl]-propranol methane sulfonate (WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H) and 4-(t-butyl)-2-(t-butylaminomethyl)-6-(4-chlorophenyl)-phenol phosphate (WR 194,965·H<sub>3</sub>PO<sub>4</sub>) after intravenous infusion to anesthetized beagles.

b. Materials and methods:

Twenty male beagles weighing 8.7 to 14.1 kg were anesthetized with 35 mg/kg sodium pentobarbital administered intravenously. Supplemental anesthesia was administered as needed. The left femoral artery and vein were catheterized for measurement of arterial pressure and drug administration, respectively. An endotracheal tube was positioned and respiration was monitored with a pneumotachometer. The Lead II electrocardiogram was recorded and its signal was fed into a cardiotachograph for determination of heart rate. All measurements were recorded on a Hewlett-Packard 7700 series recorder. Body temperature was monitored via a rectal thermistor probe and maintained near normal by application of external heat. At the termination of the experimental procedures, animals still alive were euthanized with an overdose of sodium pentobarbital.

WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H, Lot AK, was obtained from bottle number BG32210. WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H, 250 mg, was dissolved in 1 ml of absolute ethanol which was then diluted with 5% dextrose in distilled water to a final volume of 10 ml. The drug concentration in the solution was 25 mg salt/ml of vehicle. WR 194,965·H<sub>3</sub>PO<sub>4</sub>, Lot AG, was obtained from bottle number BG56327. WR 194,965·H<sub>3</sub>PO<sub>4</sub>, 250 mg, was dissolved in 2.5 ml of absolute ethanol which was then diluted with 5% dextrose in distilled water to a final volume of 25 ml. The drug concentration in the solution was 10 mg salt/ml of the vehicle.

Six of the beagles were used to determine the cumulative effect of increasing doses of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H (3 beagles) or WR 194,965·H<sub>3</sub>PO<sub>4</sub> (3 beagles) on the cardiovascular and respiratory systems. The drugs were administered as an intravenous bolus at 20 min intervals in increasing doses, and cardiovascular and respiratory responses were recorded. Progressive doses of 1 mg/kg, 1 mg/kg, 3 mg/kg, 3 mg/kg, 10 mg/kg ... were administered until death occurred. The cumulative lethal dose was recorded. Two comparisons of this data were made using the paired t test with a 5% level of significance. The first was between the baseline readings obtained prior to administration of a particular dose and the post-injection readings. The

second comparison was between the control baseline (baseline value for the 1.0 mg/kg dose) and the succeeding baseline values.

A second group of 6 beagles was used to evaluate the effects of a 30 mg/kg infusion of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H (3 beagles) and of WR 194,965·H<sub>3</sub>PO<sub>4</sub> (3 beagles) administered as a 60 min infusion. The standard 25 mg/ml solution of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H was diluted with the vehicle so that the infusion consisted of 0.5 mg salt/kg/.247 ml vehicle/min. The WR 194,965·H<sub>3</sub>PO<sub>4</sub> infusion was prepared by taking the standard 10 mg/ml solution and diluting with vehicle to obtain 0.5 mg base/kg/.764 ml vehicle/min. The respective doses were selected because they have been shown to be efficacious in the Aotus monkey screen (Schmidt, 1976). The hematocrit was monitored both during the infusion and during the 2 hr observation period following termination of the infusion. Two additional beagles were used to assess the effect of a 60 min infusion of the vehicle in a manner similar to that for the experimental animals. The paired t test of significance was used to compare baseline values with the values obtained during and after the infusion period. Values were considered significant if the computed t exceeded the tabular t value for the 5% level of significance.

A third group of 6 beagles was used to determine if WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H (3 beagles) or WR 194,965·H<sub>3</sub>PO<sub>4</sub> (3 beagles) modified the cardiovascular and respiratory effects of a series of vasoactive compounds. Epinephrine hydrochloride (2 µg/kg), isoproterenol hydrochloride (1 µg/kg), acetylcholine hydrochloride (1 µg/kg), histamine phosphate (2 µg/kg), serotonin creatinine sulfate monohydrate (10 µg/kg), norepinephrine hydrochloride (2 µg/kg), and angiotensin amide (1 µg/kg), administered intravenously as the salt, were injected at 10 min intervals and the responses monitored. After a 60 min infusion of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H, 30 mg/kg, or a 40 min infusion of WR 194,965·H<sub>3</sub>PO<sub>4</sub>, 15 mg base/kg, the series of vasoactive compounds were again injected and the responses recorded. The order of administration for these drugs was random but it was kept constant for a particular dog. The changes from control of the before- and after-infusion responses of the various drugs were analyzed with the student t test with significance set at a 5% level.

c. Results and discussion:

The cardiorespiratory responses to repeated administration of increasing doses of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H are given in Table 18. WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H at doses of 1 mg/kg or 3 mg/kg had little effect on mean arterial pressure, respiratory rate, or heart rate. However, 10 mg/kg of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H produced a significant decrease in blood pressure. This hypotension produced a transient rise in heart rate which was not significant. Moreover, the cumulative administration of 28 mg/kg WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H produced a significant negative

chronotropism as indicated by the decrease in baseline heart rate for the 30 mg/kg dose of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H when compared to the control baseline recording (the baseline for the 1.0 mg/kg injection). Additional injections of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H produced a continual decrease in the baseline heart rates. The transient hypotension associated with injection of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H appeared to be dose related as was the tachypnea which first occurred after administration of 30 mg/kg of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H and became significant with the injection of 100 mg/kg. Two of the 3 animals died after the first 100 mg/kg dose; the third animal died after the second 100 mg/kg injection. The average cumulative lethal dose was 255 mg/kg. The major terminal event was circulatory failure attributed to cardiac standstill. WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H produced disturbance in cardiac rhythm as indicated by the baseline heart rate of 51 bpm for the beagle which received a second 100 mg/kg injection of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H. Necropsy reports indicated a marked congestion of lungs, liver, and other viscera consistent with circulatory failure.

Single oral doses of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H ranging between 17.5 and 35 mg/kg have been demonstrated to be effective against both the Smith strain of Plasmodium falciparum and the Palo Alto strain of Plasmodium vivax in Aotus trivirgatus (Schmidt, 1976). However, doses of 30 mg/kg produced cardiovascular alterations when administered as an intravenous bolus injection to beagles. WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H, 30 mg/kg, was, therefore, infused in 3 beagles over a 60 min period in order to approximate the gastric absorption of an oral dose of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H. The cardiovascular and respiratory responses associated with this infusion are given in Table 19. There was little change in mean arterial pressure, respiration, or hematocrit during the infusion or the 2 hr post-infusion observation period. However, the plasma developed a reddish tint within the first 20 min of the infusion. In addition, there was a gradual decrease of heart rate during the infusion period which stabilized and became significant during the second hr post-infusion. The development of a reddish tint in the plasma and the negative chronotropism appear associated with the infusion of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H since infusion of the vehicle alone to 2 beagles did not produce these disturbances (Table 20).

The effect of an infusion of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H on the cardiorespiratory responses of a series of vasoactive drugs was assessed in a third group of beagles. The effects of the vasoactive agents before and after WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H on mean arterial pressure are given in Table 21, their effects on heart rate are given in Table 22, and their effects on respiration are given in Table 23. There were no significant alterations in the drug responses that occurred before and after the infusion of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H.

The cardiorespiratory responses to repeated administration of increasing doses of WR 194,965·H<sub>3</sub>P0<sub>4</sub> are given in Table 24. WR 194,965·H<sub>3</sub>P0<sub>4</sub>, 1 mg/kg, had little effect on mean arterial pressure, respiration or heart rate. However, 3 mg/kg produced hypotension and tachycardia. The time of onset for the tachycardia with respect to the onset of hypotension suggests that the tachycardia was a reflex response to the drug-induced hypotension. WR 194,965·H<sub>3</sub>P0<sub>4</sub> appears to have a sustained negative chronotropic effect since the baseline heart rate values for the 10 mg/kg dose of WR 194,965·H<sub>3</sub>P0<sub>4</sub> were significantly lower than the control baseline values (baseline value for 1.0 mg/kg). The hypotension observed immediately after drug injection appears transient since the blood pressure returned to baseline levels within 20 min post-injection. The 10 mg/kg dose of WR 194,965·H<sub>3</sub>P0<sub>4</sub> produced a transient tachypnea. The hypotension, tachycardia, and tachypnea show a dose-related response since 30 mg/kg increased the magnitude of change observed with 10 mg/kg. Two of the 3 animals died after the first 30 mg/kg dose; the third animal died after the second 30 mg/kg injection. The average cumulative lethal dose was 68 mg/kg. The major terminal event was a profound vasodilation. Necropsy reports indicated marked congestion of the liver, lungs, intestines, and kidneys which is consistent with circulatory failure.

Single oral doses of WR 194,965·HCl ranging between 17.5 and 35 mg/kg of the base have been reported effective against both the Smith strain of Plasmodium falciparum and the Palo Alto strain of Plasmodium vivax infections in Aotus trivirgatus (Schmidt, 1976). However, doses of 10 or 30 mg/kg produced marked cardiorespiratory alterations when administered as an intravenous bolus injection to beagles. WR 194,965·H<sub>3</sub>P0<sub>4</sub>, 30 mg base/kg, was infused into 3 beagles over a 60 min period to approximate the gastric absorption of an oral dose of WR 194,965·H<sub>3</sub>P0<sub>4</sub>. The cardiovascular and respiratory responses associated with the infusion are given in Table 25. WR 194,965·H<sub>3</sub>P0<sub>4</sub>, 30 mg base/kg, administered as an intravenous infusion over one hr produced no significant alterations in blood pressure, respiration, or hematocrit. However, there was a progressive decrease in heart rate and the plasma developed a reddish tint during the infusion of WR 194,965·H<sub>3</sub>P0<sub>4</sub> which continued during the 2 hr observation period. These effects appear to be due to WR 194,965·H<sub>3</sub>P0<sub>4</sub> since infusion of the vehicle in 2 beagles had little effect on these parameters (Table 20). A lower dose of WR 194,965·H<sub>3</sub>P0<sub>4</sub>, 15 mg base/kg, was administered as an infusion to 3 beagles. The cardiovascular and respiratory effects of this infusion are given in Table 26. WR 194,965·H<sub>3</sub>P0<sub>4</sub> again produced a significant decrease in heart rate although the onset was later. This could be attributed to the slower infusion rate used in this study or to the bilateral vagotomy performed on these animals. As in the one hr infusion, WR 194,965·H<sub>3</sub>P0<sub>4</sub> tended to decrease respiratory rate, but not significantly. The infusion studies support the

preliminary observations made during the cumulative dosing regimen that WR 194,965·H<sub>3</sub>PO<sub>4</sub> has a long-lasting negative chronotropic effect. The hypotension associated with the bolus injection of WR 194,965·H<sub>3</sub>PO<sub>4</sub> may be related to the direct depressant effects on the arterioles at the initial higher concentrations of a bolus injection which are not present during the slow infusion.

The effect of an infusion of WR 194,965·H<sub>3</sub>PO<sub>4</sub> on the cardiovascular and respiratory responses of vasoactive drugs was assessed in the third group of beagles. The effects of the vasoactive agents before and after WR 194,965·H<sub>3</sub>PO<sub>4</sub> on mean arterial pressure are given in Table 27, their effects on heart rate are given in Table 28, and their effects on respiration are given in Table 29. There were no significant alterations in the drug responses that occurred before and after the infusion of WR 194,965·H<sub>3</sub>PO<sub>4</sub>.

In conclusion, WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H, when administered as an intravenous bolus, produced an immediate hypotension and tachypnea. There was a more slowly developing negative chronotropism associated with the cumulative administration of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H. Death occurred after an average cumulative dose of 255 mg/kg. Infusions of 30 mg/kg had little effect on respiration or mean arterial pressure but did produce a gradual slowing of heart rate that became significant during the 2 hr post-infusion observation period. Infusion of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H had little effect on the cardiovascular and respiratory effects of epinephrine, isoproterenol, norepinephrine, acetylcholine, angiotensin, serotonin and histamine.

WR 194,965·H<sub>3</sub>PO<sub>4</sub>, when administered as an intravenous bolus, produced a transient hypotension, tachypnea, and a reflex tachycardia. There was a long term negative chronotropism associated with administration of WR 194,965·H<sub>3</sub>PO<sub>4</sub>. Death occurred after an average cumulative dose of 68 mg/kg. Infusions of 30 mg/kg of the WR 194,965 base had little effect on respiration or mean arterial pressure but produced a marked negative chronotropism which was still present 2 hr post-infusion. Infusion of 15 mg/kg of the WR 194,965 base had little effect on the cardiovascular and respiratory actions of epinephrine, isoproterenol, norepinephrine, angiotensin, acetylcholine, serotonin, and histamine.

#### 6. Normal phase solvents for high pressure liquid chromatographic quantitation of experimental antimalarials.

##### a. Background:

The increasing need for information on the bioavailability and pharmacokinetic properties of experimental antimalarial drugs has led to a demand for the fast and accurate quantitation of drug

levels in biological fluids. The accurate determination of these levels requires a process of separation and isolation of the drug from interfering materials followed by an analysis of the isolated fractions. The recently developed method of high pressure liquid chromatography (HPLC) is capable of rapid quantitation of drug levels from simple extracts of the biological samples. However, the conditions of use under which the technique can be relied upon for precise, accurate results must often be carefully controlled.

b. Materials and methods:

A Water's High Performance Liquid Chromatograph equipped with a 280 nm absorbance detector was utilized throughout the experimental trials. Ethyl acetate extracts of whole blood samples were evaporated to dryness and then reconstituted in an appropriate volume of the mobile phase. Small aliquots (5 to 50  $\mu$ l) of this final solution were chromatographed on a column (4 mm I.D. x 30 cm) of 10  $\mu$  fully porous silica bonded with a monomolecular layer of cyanopropylsilane.

A calibration curve was constructed using internal standards. WR 184,806 dissolved in methanol is the internal standard for WR 142,490 and WR 180,409; the internal standard for the analysis of WR 184,806 is WR 142,490 dissolved in methanol. Duplicate or triplicate injections (depending on the number of "unknown" samples) were made to yield the data to construct the linear calibration curve relating peak height ratio of WR 142,490 to WR 184,806 or WR 180,409 compared to the amount of the standard injected.

A mobile phase composed primarily of hexane (47%) and ethylene dichloride (47%) combined with very limited amounts of water (0.1%), formic acid (0.4%) and acetonitrile (5.5%) made it possible to separate antimalarials from major interference peaks while maintaining the required separation from the internal standard. This mobile phase has been modified for the analysis of blood samples of WR 142,490, WR 180,409 and WR 184,806. In addition, this mobile phase has been modified successfully in preliminary studies of WR 172,435, WR 171,669 and WR 30,090.

WR 30,090 is a very lipid soluble, hydrophobic antimalarial for which no acceptable analytical procedure is currently available. The preliminary effort in this laboratory was to extend the basic extraction and chromatographic technique to the analysis of WR 30,090. In the general procedure a known amount of the internal standard (WR 29,656.HCl) in 50  $\mu$ l of methanol was added to each 5 ml blood sample and the WR 30,090 "spike" was then added. The spiked blood was allowed to equilibrate on a shaker for 30 min. To each 5 ml blood sample was added 5 ml of pH 7.4 phosphate buffer and the 10 ml sample extracted 3 times by shaking 30 min with 10 ml aliquots

each of ethyl acetate and centrifuging. The combined ethyl acetate extracts of each blood sample were evaporated to dryness in a 45 ml glass conical tube under a gentle stream of filtered, dried air at 40°C. Each sample residue was dissolved in 5.0 ml of the multi-component solvent system. A small aliquot (50  $\mu$ l) of this sample solution was injected onto a Water's microbondapak column and allowed to elute at the low flow rate of 2.0 ml/min. In this preliminary study a simple ratio of peak heights of the "unknown" peak and the internal standard was used for quantitative analysis.

A comparative crossover bioavailability study on WR 180,409-H<sub>3</sub>PO<sub>4</sub> was carried out in 4 healthy, mature, registered male beagle dogs, fasted for 24 hr prior to drug administration. The capsule formulation (250 mg, Lot E521, developed by Lafayette Pharmacal) or tableted formulation (250 mg, Lot D522, INTERx Corporation) was administered orally by means of a balling gun. Five ml blood samples were withdrawn at 0, 1, 2, 3, 4, 6, 8, 10, 12, 24, 48 and 72 hr and at 4, 7, 10 and 14 days after dosing. After 14 days the formulation administration schedule was reversed so that the dogs which received the tablet during the initial drug administration were presented with a capsule during the second drug administration and vice-versa.

The blood samples were analyzed for the concentration of WR 180,409 by means of high pressure liquid chromatography using the aforementioned mobile phase and WR 184,806 as an internal standard.

c. Results and discussion:

This versatile mobile phase HPLC technique has been used to analyze human blood samples for 2 drugs. Approximately 1600 blood samples were analyzed for WR 142,490 levels and approximately 250 blood samples were analyzed for WR 184,806 levels. The results of these studies are being mathematically analyzed to determine pharmacokinetic parameters for these drugs in humans.

In support of the WR 180,409 bioavailability study, approximately 300 dog blood samples have been analyzed for WR 180,409 levels. In one of 4 dogs there was no significant difference in the rate of absorption and maximum blood level achieved by either the capsule or the tablet formulation.

In another dog there was a significant difference in both the rate of absorption and the maximum blood level achieved after drug administration. The apparent absorption of WR 180,409 from the capsule was complete within 6 hr, achieving a maximum blood level which was 2 times greater than the blood level attained after tablet

administration. Maximum absorption from the tablet required 24 hr in this animal.

In the remaining two animals the rates of absorption of WR 180,409 was nearly the same for both the tablet and the capsule; however, the capsuled formulation achieved a blood level which was nearly 3 times greater than that of the tablet. Thus, in 3 of 4 dogs the bioavailability of WR 180,409 was significantly greater after oral administration of the capsuled preparation than the same dose administered by means of a tableted formulation.

Table 30 presents the results of the preliminary experiments on WR 30,090 quantitation. The percent error of 11 samples ranged from 0.1% to 6.0%. Although more work needs to be done to define the limits of accuracy and precision, these results are very encouraging.

#### 7. Development of new antimalarial drugs.

##### a. Background:

The Department of Pharmacology is also charged with the responsibility of writing Notice of Claimed Investigational Exemption for New Drug (IND) submissions. These include planning and designing the experiments, and assembling, evaluating, coordinating and correlating the data required for both the initial submission and all supplementary submissions for each drug. The data must be continuously monitored and evaluated from both in-house and contract sources, as well as proprietary and open literature sources.

##### b. Investigational New Drug submissions:

Four new IND applications were written. They were WR 149,024-HCl, WR 172,435-CH<sub>3</sub>SO<sub>3</sub>H, WR 194,965-H<sub>3</sub>PO<sub>4</sub> and "TAB."

Five supplements to IND submissions were written. They were for 5 single drugs.

##### c. Technical monitoring of contracts necessary for data generation:

Twelve active contracts were closely guided by the Department. These ranged from pharmacological areas such as toxicology, drug metabolism and bioavailability of the drugs to those of their formulation and development of methods to determine blood levels of drugs.

Table 1  
 Percent of WR 172,435-<sup>14</sup>C Derived Radioactivity Recovered in  
 the Urine and Feces of Mice after Oral Administration of the Drug<sup>a</sup>

Time (hr)	Percent <sup>14</sup> C Recovered <sup>b</sup>	
	Urine	Feces
0-12	0.14	58.00
12-24	0.05	4.40
24-36	0.09	4.00
36-48	0.04	1.40
48-60	0.07	1.50
60-72	0.03	0.80
72-84	0.02	0.90
84-96	0.04	0.40
96-108	0.02	0.90
108-120	0.08	0.50
120-144	0.06	1.30
144-168	0.04	1.20
168-192	0.04	1.10
Cumulative Recovery	0.72	76.40

<sup>a</sup>WR 172,435-<sup>14</sup>C was administered 20 mg/kg orally.

<sup>b</sup>Average of two cages of 4 mice each.

Table 2  
 TLC Analysis of Percent Apparent Parent Drug  
 in Feces After Oral Administration of WR 172,435-<sup>14</sup>C to Mice<sup>a</sup>

Time (hr)	% D <sup>b</sup>
0-12	43.0
12-24	36.4
24-36	38.7
36-48	48.6
48-60	40.2

<sup>a</sup>WR 172,435-<sup>14</sup>C was administered 20 mg/kg orally.

<sup>b</sup>The area of radioactivity with R<sub>f</sub> value corresponding to the standard for WR 172,435-<sup>14</sup>C on TLC using n-butanol:acetic acid: water (10:1:1 by volume) as solvent system.

Table 3  
 Concentration of  $^{14}\text{C}$ -Drug Equivalents in the Plasma and RBCs of  
 the Mouse after a Single Oral Dose of WR 172,435- $^{14}\text{C}\cdot\text{CH}_3\text{SO}_3\text{H}^{\text{a}}$

Time (hr) <sup>b</sup>	$^{14}\text{C}$ -Drug Equivalents ( $\mu\text{g}/\text{ml}$ )	
	Plasma	RBC <sup>c</sup>
0.5	0.54	1.26
1.0	1.09	1.48
1.5	2.19	0.77
2.0	2.02	1.11
4.0	1.09	0.74
6.0	0.44	0.56

<sup>a</sup>WR 172,435- $^{14}\text{C}$  was administered 20 mg/kg orally.

<sup>b</sup>Four mice per time period with a single determination from their pooled blood.

<sup>c</sup>Radioactivity for plasma samples was determined directly, for red blood cells it was calculated from the following equation:

$$\text{RBC } \mu\text{g}/\text{ml} = \frac{\text{Whole blood } (\mu\text{g}/\text{ml}) - [\text{Plasma } (\mu\text{g}/\text{ml}) \times (1 - \text{Hct})]}{\text{Hct}}$$

**Table 4**  
**Distribution of Radioactivity Derived from WR 172,435-<sup>14</sup>C**  
**in Tissues of Mice after a Single Dose of the Drug<sup>a</sup>**

Tissue <sup>b</sup>	μg/g (% <sup>d</sup> )				120 hr <sup>c</sup>
	2 hr <sup>c</sup>	6 hr <sup>c</sup>	24 hr <sup>c</sup>	72 hr <sup>c</sup>	
Brain	0.20 (0)	0.16 (0)	0.80 (0.1)	1.07 (0.1)	0.85 (0.1)
Eyes	0.33 (0)	3.03 (0)	7.81 (0.1)	25.08 (0.3)	13.91 (0.2)
Submaxillary Salivary Glands	2.51 (0.5)	6.32 (0.2)	10.72 (0.3)	10.27 (0.3)	8.67 (0.3)
Heart	5.67 (0.2)	4.36 (0.3)	9.00 (0.2)	4.79 (0.1)	3.50 (0.1)
Lungs	11.80 (0.4)	26.59 (0.8)	27.24 (1.0)	15.49 (0.5)	11.14 (0.4)
Gall Bladder + Bile	3729.40 (3.8)	44.75 (0.1)	6.80 (0)	27.04 (0)	5.34 (0)
Liver	34.19 (12.7)	44.28 (13.4)	22.00 (6.1)	10.50 (2.7)	6.81 (2.1)
Adrenal Glands	58.60 (0.2)	66.20 (0.2)	1.33 (0)	81.21 (0.2)	18.08 (0)
Kidney	7.40 (0.6)	19.00 (1.5)	23.17 (2.0)	11.84 (1.0)	11.16 (0.9)
Spleen	13.75 (0.3)	18.70 (0.3)	18.56 (0.4)	6.18 (0.2)	8.16 (0.2)
G.I. Tract	132.83 (81.2)	92.57 (52.8)	34.12 (22.2)	5.12 (2.5)	4.79 (2.9)
Abdominal Fat	2.01 (0.3)	4.27 (0.8)	6.88 (1.2)	16.59 (2.1)	12.27 (1.5)
Skeletal Muscle	1.39 (0.1)	3.38 (0.1)	4.24 (0.2)	3.43 (0.1)	-- <sup>e</sup>

Footnotes to Table 4

aWR 172,435-<sup>14</sup>C was administered 20 mg/kg orally.

bMethanolic extracts.

cHours after dosing at sacrifice, 4 mice per time period.

dPercent of administered dose.

eNo sample taken.

Table 5  
 Percent of WR 177,602-<sup>14</sup>C Derived Radioactivity Recovered in the  
 Urine and Feces of Mice after Oral Administration of the Drug<sup>a</sup>

Time (hr)	Percent <sup>14</sup> C Recovered <sup>b</sup>	
	Urine	Feces
0-12	7.96	12.47
12-24	0.78	11.35
24-36	8.52	26.31
36-48	2.28	8.06
48-60	1.81	6.01
60-72	0.85	2.35
72-96	0.71	2.11
96-144	0.41	0.82
144-168	<u>0.06</u>	<u>0.23</u>
Cumulative Recovery	23.38	69.71

<sup>a</sup>WR 177,602-<sup>14</sup>C was administered 10 mg/kg orally.

<sup>b</sup>Average of two cages of 4 mice each.

Table 6  
 Distribution of Radioactivity Derived from WR 177,602-<sup>14</sup>C  
 in Tissues of Mice 168 Hours after a Single Dose of the Drug<sup>a</sup>

Tissue <sup>b</sup>	% of Administered Dose	$\mu\text{g/g}$ <sup>c</sup>
Brain	0.02	0.03
Eyes	0.01	0.55
Submaxillary Salivary Glands	0.01	0.03
Heart	0.02	0.43
Lungs	0.03	0.25
Gall Bladder + Bile	0.01	145.93
Liver	0.19	0.03
Adrenal Glands	0.01	9.02
Kidney	0.03	0.09
Spleen	0.01	0.40
G.I. Tract	0.11	0.03
Abdominal Fat	--	0.03
Skeletal Muscle	--	0.48

<sup>a</sup>WR 177,602-<sup>14</sup>C was administered 10 mg/kg orally.

<sup>b</sup>Methanolic extracts.

<sup>c</sup>Average of two groups, 4 mice per group, calculated as drug-equivalents.

Table 7

Cumulative Effect of WR 177,602·HCl, WR 142,490·HCl or Drug Vehicle on  
Mean Arterial Pressure in Anesthetized Beagles<sup>a</sup>

Dose of Antimalarial (Volume of Vehicle) <sup>b</sup>	WR 177,602·HCl		WR 142,490·HCl		Vehicle <sup>c</sup>	
	Baseline	Post-Injection	Baseline	Post-Injection	Baseline	Post-Injection
1 mg/kg (0.2 cc)	131.7 ± 6.6	128.0 ± 7.6 <sup>d</sup>	124.3 ± 6.8	120.0 <sup>d</sup> ± 6.5	121.3 ± 5.3	121.0 ± 6.4
3 mg/kg (0.6 cc)	134.5 ± 4.6	108.7 ± 6.9 <sup>d</sup>	122.5 ± 7.0	72.8 <sup>d</sup> ± 8.6	137.0 ± 3.9	138.0 ± 3.9
10 mg/kg (2.0 cc)	126.3 ± 9.4	66.5 ± 9.4 <sup>d</sup>	121.6 <sup>f</sup> ± 11.7 <sup>f</sup>	42.0 <sup>d</sup> ± 8.7	127.0 ± 6.0	127.3 ± 5.7
30 mg/kg (6.0 cc)	98.5 ± 4.5 <sup>e,g</sup>	55.5 ± 32.5	--	--	123.0 <sup>h</sup> ± 6.8	127.0 ± 7.0

<sup>a</sup>Values are mean ± SEM in mmHg.

<sup>b</sup>Doses were administered as an i.v. bolus at 20 minute intervals. Each dose was administered twice. Baseline readings were made immediately prior to the appropriate drug injection. Post-injection readings were made during maximum response.

<sup>c</sup>Vehicle was a 1:1:3 solution of ethanol:propylene glycol:isotonic saline.

<sup>d</sup>Significantly different from appropriate baseline (paired t test, 5% level of significance).

Footnotes to Table 7 (cont.)

<sup>e</sup>Significantly different from the 1.0 mg/kg baseline value (Dunnett's test, 5% level of significance).

<sup>f</sup>This dose was only given once because the three beagles in this group expired prior to the next injection. The average cumulative lethal dose of WR 142,490·HCl was 18 mg/kg.

<sup>g</sup>One beagle expired after the second 10 mg/kg dose; these values are for the remaining beagles. The average cumulative lethal dose of WR 177,602·HCl was 49 mg/kg.

<sup>h</sup>This volume of vehicle was administered only once to the three beagles in this group.

Table 8  
Cumulative Effect of WR 177,602·HCl, WR 142,490·HCl or Drug Vehicle on  
Heart Rate in Anesthetized Beagles<sup>a</sup>

Dose of Antimalarial (Volume of Vehicle) <sup>b</sup>	WR 177,602·HCl		WR 142,490·HCl		Vehicle <sup>c</sup>		
	Baseline	Post-Injection	Baseline	Post-Injection	Baseline	Post-Injection	
1 mg/kg (0.2 cc)	158.3 + 3.1	+ 8.9	163.7 + 10.3	+ 8.9	180.7 + 8.9	+ 5.9	138.2 + 6.5
3 mg/kg (0.6 cc)	145.5 + 3.1	- 6.1 <sup>d</sup>	164.8 + 8.3	+ 157.2 + 18.4	125.5 + 18.4	+ 13.1	150.7 + 13.0
10 mg/kg (2.0 cc)	132.2 + 10.9 <sup>e</sup>	+ 4.9 <sup>d</sup>	84.3 + 6.5 <sup>f</sup>	+ 145.6 <sup>f</sup> + 9.5 <sup>d</sup>	71.3 <sup>d</sup> + 9.5 <sup>d</sup>	+ 6.1	153.5 + 11.0
30 mg/kg (6.0 cc)	109.5 + 15.5 <sup>e,g</sup>	+ 14.5	--	--	148.0 <sup>h</sup> + 6.4 <sup>h</sup>	+ 4.7	145.3 + 4.7

<sup>a</sup>Values are mean + SEM in bpm.

<sup>b</sup>Doses were administered as an i.v. bolus at 20 minute intervals. Each dose was administered twice. Baseline readings were made immediately prior to the appropriate drug injection. Post-injection readings were made during maximum response.

<sup>c</sup>Vehicle was a 1:1:3 solution of ethanol:propylene glycol:isotonic saline.

<sup>d</sup>Significantly different from appropriate baseline (paired t test, 5% level of significance).

Footnotes to Table 8 (cont.)

<sup>e</sup>Significantly different from the 1.0 mg/kg baseline value (Dunnett's test, 5% level of significance).

<sup>f</sup>This dose was only given once because the three beagles in this group expired prior to the next injection. The average cumulative lethal dose for WR 142,490-HCl was 18 mg/kg.

<sup>g</sup>One beagle expired after the second 10 mg/kg dose; these values are for the remaining two beagles. The average cumulative lethal dose of WR 177,602-HCl was 48 mg/kg.

<sup>h</sup>This volume of vehicle was administered only once to the three beagles in this group.

Table 9  
Cumulative Effect of WR 177,602·HCl, WR 142,490·HCl or Drug Vehicle on  
Respiratory Rate in Anesthetized Beagles<sup>a</sup>

Dose of Antimalarial (Volume of Vehicle) <sup>b</sup>	WR 177,602·HCl			WR 142,490·HCl			Vehicle <sup>c</sup>	
	Baseline	Post-Injection	Baseline	Post-Injection	Baseline	Post-Injection	Baseline	Post-Injection
1 mg/kg (0.2 cc)	11.0 + 1.4	10.0 + 1.2	6.8 + 0.9	8.3 + 1.9	6.8 + 1.1	7.0 + 1.3		
3 mg/kg (0.6 cc)	10.7 + 1.5	13.8 + 2.9	7.8 + 1.5	41.2 <sup>d</sup> + 9.7	8.3 + 1.1	8.5 + 1.2		
10 mg/kg (2.0 cc)	34.5 + 13.5 <sup>e</sup>	66.0 <sup>d</sup> + 5.8	12.0 <sup>f</sup> + 2.6	84.0 <sup>d</sup> + 6.9	9.8 + 2.6	10.2 + 2.8		
30 mg/kg (6.0 cc)	69.0 + 3.0 <sup>e,g</sup>	51.0 + 15.0	--	--	9.0 <sup>h</sup> + 3.1 <sup>h</sup>	7.0 + 2.6		

<sup>a</sup>Values are mean  $\pm$  SEM in respirations per minute.

<sup>b</sup>Doses were administered as an i.v. bolus at 20 minute intervals. Each dose was administered twice. Baseline readings were made immediately prior to the appropriate drug injection. Post-injection readings were made during maximum response.

<sup>c</sup>Vehicle was a 1:1:3 solution of ethanol:propylene glycol:isotonic saline.

<sup>d</sup>Significantly different from appropriate baseline (paired  $t$  test, 5% level of significance).

Footnotes to Table 9 (cont.)

eSignificantly different from the 1.0 mg/kg baseline value (Dunnett's test, 5% level of significance).

fThis dose was only given once because the three beagles in this group expired prior to the next injection. The average cumulative lethal dose for WR 142,490·HCl was 18 mg/kg.

gOne beagle expired after the second 10 mg/kg dose; these values are for the remaining two beagles. The average cumulative lethal dose of WR 177,602·HCl was 48 mg/kg.

hThis volume of vehicle was administered only once to the three beagles in this group.

Table 10

Heart Rate Responses of Anesthetized Beagles to a 60 Minute Infusion of WR 177,602·HCl, WR 142,490·HCl or Drug Vehicle<sup>a</sup>

	Time - Minutes						Observation Period	
	Infusion Period			90				
	<u>0</u>	<u>20</u>	<u>40</u>	<u>60</u>	<u>90</u>	<u>120</u>	<u>150</u>	<u>180</u>
WR 177,602·HCl 35 mg/kg	162.0 + 19.4	166.0 + 16.7	159.0 + 12.0	151.0 + 9.0	147.0 + 12.2	146.0 + 13.7	147.0 + 12.2	156.0 + 10.2
WR 142,490·HCl 35 mg/kg	160.0 + 14.0	146.0 + 15.5	141.0 + 13.8	131.0 + 13.9	127.0 + 12.5	127.0 + 12.5	122.0 + 14.3	119.0 + 12.0
Drug Vehicle <sup>b</sup>	135.0 + 18.2	151.0 + 8.7	149.0 + 13.3	159.0 + 12.0	168.0 + 10.7	158.0 + 7.7	156.0 + 10.0	140.0 + 12.9

<sup>a</sup>Values are the mean  $\pm$  SEM from three beagles. Units are bpm.

<sup>b</sup>Drug vehicle is a 1:1:3 solution of ethanol:propylene glycol:isotonic saline infused at 0.97 ml/min.

Table 11

Mean Arterial Pressure Responses of Anesthetized Beagles to a  
60 Minute Infusion of WR 177,602-HCl, WR 142,490-HCl or Drug Vehicle<sup>a</sup>

	Infusion Period				Observation Period			
	0	20	40	60	90	120	150	180
WR 177,602-HCl 35 mg/kg	118.0 + 13.5	115.6 + 5.9	121.0 + 4.4	119.0 + 2.1	121.0 + 7.2	115.0 + 3.6	112.0 + 4.6	120.0 + 14.0
WR 142,490-HCl 35 mg/kg	136.0 + 9.8	123.0 + 9.6	131.3 + 8.1	135.0 + 7.3	134.0 + 9.0	128.0 + 11.0	126.0 + 13.7	128.0 + 11.9
Drug Vehicle <sup>b</sup>	121.0 + 6.7	123.0 + 6.7	122.0 + 7.0	134.0 + 4.7	130.0 + 2.3	129.0 + 5.8	119.0 + 2.0	119.0 + 7.1

<sup>a</sup>Values are the mean  $\pm$  SEM from three beagles. Units are mmHg.

<sup>b</sup>Drug vehicle is a 1:1:3 solution of ethanol:propylene glycol:isotonic saline infused at 0.97 ml/min.

Table 12

Respiratory Responses of Anesthetized Beagles to a 60 Minute Infusion of WR 177,602·HCl, WR 142,490·HCl or Drug Vehicle<sup>a</sup>

	Time - Minutes						Observation Period
	0	20	40	60	90	120	
WR 177,602·HCl 35 mg/kg	10.6 + 2.6	9.6 + 2.2	10.3 + 1.3	10.6 + 1.2	15.0 + 4.2	12.0 + 1.7	12.6 + 1.2
WR 142,490·HCl 35 mg/kg	12.3 + 3.4	9.6 + 2.7	9.3 + 2.3	11.0 + 2.6	10.0 + 1.5	9.6 + 1.8	11.3 + 2.0
Drug Vehicle <sup>b</sup>	12.0 + 2.9	10.0 + 1.5	8.3 + 0.7	8.6 + 2.6	9.7 + 2.4	10.0 + 4.1	8.3 + 3.9

<sup>a</sup>Values are the mean + SEM from three beagles. Units are respirations per minute.

<sup>b</sup>Drug vehicle is a 1:1:3 solution of ethanol:propylene glycol:isotonic saline infused at 0.97 ml/min.

Table 13  
 Cardiovascular and Respiratory Responses of Anesthetized Beagles  
 to 20 Minute Infusions of WR 177,602·HCl or WR 142,490·HCl, 35 mg/kg<sup>a</sup>

	WR 177,602·HCl		WR 142,490·HCl	
	0 min	20 min	0 min	20 min
Heart Rate (bpm)	165.3 + 14.8	123.3 <sup>b</sup> + 15.9	171.7 + 11.7	128.3 <sup>b</sup> + 7.3
Mean Arterial Pressure (mmHg)	110.7 + 4.3	107.0 + 6.1	122.0 + 7.8	98.7 <sup>b</sup> + 10.7
Respiratory Rate (respirations/min)	9.7 + 1.3	12.7 + 2.0	15.0 + 6.4	34.7 + 9.5

<sup>a</sup>Values are mean  $\pm$  SEM for 3 beagles.

<sup>b</sup>Significantly different from 0 min baseline (paired  $t$  test, 5% level of significance).

Table 14

Venous Hematocrit of Anesthetized Beagles during and after a 60 Minute Infusion of WR 177,602·HCl, WR 142,490·HCl or Drug Vehicle<sup>a</sup>

		Time - Minutes				Observation Period
		0	20	40	60	
WR 177,602·HCl <sup>b</sup> (35 mg/kg)	44.0 + 3.2	44.3 + 3.5	45.3 + 4.7	44.3 + 3.3	45.0 + 3.5	44.0 + 3.1
WR 142,490·HCl <sup>c</sup> (35 mg/kg)	43.3 + 2.0	44.3 + 1.3	44.0 + 1.5	43.3 + 1.3	43.3 + 1.3	45.0 + 3.1
Drug Vehicle <sup>d</sup> (0.97 ml/min)	43.7 + 2.6	45.0 + 4.0	46.7 + 4.5	47.2 + 4.6	48.3 + 5.2	47.5 + 4.8

<sup>a</sup>Values are mean  $\pm$  SEM expressed as % of blood volume.

<sup>b</sup>A slight reddish tint of the plasma was observed in two beagles.

<sup>c</sup>The plasma from all three beagles had a reddish coloration.

<sup>d</sup>Drug vehicle (1:1:3 ethanol:propylene glycol:isotonic saline) did not discolor plasma of beagles.

Table 15  
Heart Rate Responses of Anesthetized Beagles to Vasoactive Compounds  
Both before and after a 20 Minute Intravenous Infusion of WR 177,602·HCl or WR 142,490·HCl, 35 mg/kg<sup>a</sup>

Vasoactive Compound ( $\mu$ g/kg - salt)	WR 177,602·HCl		WR 142,490·HCl	
	Control	Post-Infusion	Control	Post-Infusion
Phenylephrine - 3	80.3 ± 5.2 <sup>b</sup>	102.0 ± 11.1	86.7 ± 3.0 <sup>b</sup>	85.3 ± 8.7
- 10	70.7 ± 6.7 <sup>b</sup>	90.0 ± 3.8 <sup>b</sup>	98.0 ± 14.3	95.3 ± 2.4
Isoproterenol - 0.5	157.7 ± 10.8 <sup>b</sup>	158.3 ± 7.5 <sup>b</sup>	131.3 ± 11.3 <sup>b</sup>	135.7 ± 7.6 <sup>b</sup>
- 1.0	178.0 ± 43.5 <sup>b</sup>	170.0 ± 22.1 <sup>b</sup>	142.3 ± 11.6 <sup>b</sup>	138.0 ± 5.0 <sup>b</sup>
Angiotensin - 0.5	96.0 ± 5.8	112.7 ± 16.7	114.3 ± 4.7	115.7 ± 10.3
- 1.0	119.0 ± 7.8	117.7 ± 6.4	125.7 ± 15.5	104.3 ± 8.2
Acetylcholine - 1.0	123.7 ± 6.2 <sup>b</sup>	112.7 ± 1.7 <sup>b</sup>	106.3 ± 11.6	109.7 ± 2.0 <sup>b</sup>
- 2.0	118.0 ± 5.9	114.7 ± 3.9	114.7 ± 5.0 <sup>b</sup>	111.0 ± 8.0
Serotonin - 10.0	89.3 ± 7.7	104.7 ± 7.9	97.3 ± 10.7	97.0 ± 6.0
- 25.0	84.0 ± 5.7	94.7 ± 6.7	84.7 ± 1.9 <sup>b</sup>	85.0 ± 6.1
Histamine - 1.0	102.3 ± 1.2	103.7 ± 0.3 <sup>b</sup>	99.7 ± 6.1	91.0 ± 12.2
- 2.0	97.7 ± 4.9	104.7 ± 4.6	90.0 ± 5.6	107.3 ± 8.1

<sup>a</sup>Values are mean + SEM for three beagles. Values are the maximum percentage response compared to the pre-injection baseline for each dosage of vasoactive compound.

<sup>b</sup>Vasoactive compound caused significant change from baseline (paired *t* test, 5% level of significance).

**Table 16**  
**Mean Arterial Pressure Responses of Anesthetized Beagles to Vasoactive Compounds**  
**Both before and after a 20 Minute Intravenous Infusion of WR 177,602·HCl or WR 142,490·HCl, 35 mg/kg<sup>a</sup>**

Vasoactive Compound ( $\mu$ g/kg - salt)	WR 177,602·HCl		WR 142,490·HCl	
	Control	Post-Infusion	Control	Post-Infusion
Phenylephrine - 3	135.0 ± 10.5	130.0 ± 7.8 <sup>b</sup>	138.3 ± 2.2 <sup>b</sup>	123.7 ± 3.7 <sup>b,c</sup>
- 10	159.0 ± 10.2 <sup>b</sup>	120.7 ± 4.1 <sup>b,c</sup>	139.7 ± 1.9 <sup>b</sup>	115.3 ± 0.3 <sup>b,c</sup>
Isoproterenol - 0.5	63.0 ± 5.1 <sup>b</sup>	64.7 ± 7.9 <sup>b</sup>	60.0 ± 6.1 <sup>b</sup>	77.3 ± 9.4 <sup>b</sup>
- 1.0	57.7 ± 4.7 <sup>b</sup>	59.3 ± 4.8 <sup>b</sup>	47.3 ± 4.7 <sup>b</sup>	59.3 ± 7.5 <sup>b</sup>
Angiotensin - 0.5	157.7 ± 21.2	131.3 ± 2.4 <sup>b</sup>	161.0 ± 8.3 <sup>b</sup>	126.3 ± 9.8 <sup>b</sup>
- 1.0	169.0 ± 11.0 <sup>b</sup>	141.0 ± 8.2 <sup>b</sup>	194.7 ± 4.9 <sup>b</sup>	150.3 ± 6.9 <sup>b,c</sup>
Acetylcholine - 1.0	67.0 ± 4.9 <sup>b</sup>	65.0 ± 5.3 <sup>b</sup>	67.7 ± 10.7 <sup>b</sup>	67.3 ± 5.6 <sup>b</sup>
- 2.0	54.0 ± 3.1	54.3 ± 5.5 <sup>b</sup>	65.3 ± 6.7 <sup>b</sup>	65.0 ± 5.0 <sup>b</sup>
Serotonin - 10.0	72.0 ± 3.8 <sup>b</sup>	83.0 ± 13.9	64.0 ± 5.8 <sup>b</sup>	86.7 ± 13.6
- 25.0	85.7 ± 18.7	100.0 ± 22.0	97.3 ± 25.4	100.0 ± 11.0
Histamine - 1.0	60.0 ± 4.5 <sup>b</sup>	67.0 ± 1.0 <sup>b</sup>	64.0 ± 9.5 <sup>b</sup>	72.3 ± 4.2 <sup>b</sup>
- 2.0	58.3 ± 1.9 <sup>b</sup>	56.0 ± 1.5 <sup>b</sup>	58.7 ± 6.4 <sup>b</sup>	53.7 ± 0.7 <sup>b</sup>

<sup>a</sup>Values are mean + SEM for three beagles. Values are the maximum percentage response compared to the pre-injection baseline for each dosage of vasoactive compound.

<sup>b</sup>Vasoactive compound caused significant change from baseline (paired *t* test, 5% level of significance).

<sup>c</sup>Maximum percentage response post WR 177,602·HCl or WR 142,490·HCl infusion is significantly different from control responses (student *t* test, 5% level of significance).

Table 17  
Respiratory Responses of Anesthetized Beagles to Vasoactive Compounds  
Both before and after a 20 Minute Intravenous Infusion of WR 177,602·HCl or WR 142,490·HCl, 35 mg/kg<sup>a</sup>

Vasoactive Compound ( $\mu\text{g}/\text{kg}$ - salt)	WR 177,602·HCl		WR 142,490·HCl	
	Control	Post-Infusion	Control	Post-Infusion
Phenylephrine - 3.0	64.3 ± 12.2	79.3 ± 5.8	74.3 ± 3.8 <sup>b</sup>	74.0 ± 12.1 <sup>b</sup>
- 10.0	91.0 ± 14.8	69.0 ± 15.5	90.3 ± 9.7	91.3 ± 4.7 <sup>b</sup>
Isoproterenol - 0.5	106.0 ± 10.1	150.0 ± 10.3 <sup>b,c</sup>	114.3 ± 11.0	97.3 ± 11.9
- 1.0	155.0 ± 8.7 <sup>b</sup>	152.7 ± 20.5 <sup>b</sup>	154.3 ± 23.4	95.3 ± 18.6
Angiotensin - 0.5	87.3 ± 12.7	101.0 ± 19.9	139.0 ± 27.5	97.0 ± 12.7
- 1.0	134.0 ± 40.0	117.7 ± 36.1	140.3 ± 17.1	104.3 ± 13.7
Acetylcholine - 1.0	114.7 ± 20.9	105.0 ± 5.0	116.0 ± 1.5 <sup>b</sup>	97.3 ± 17.3
- 2.0	113.0 ± 20.3	129.0 ± 11.2	144.0 ± 13.1	118.3 ± 18.6
Serotonin - 10.0	132.0 ± 16.4	182.7 ± 16.8 <sup>b</sup>	99.0 ± 11.5	141.3 ± 18.6
- 25.0	148.0 ± 33.6	231.0 ± 26.9 <sup>b</sup>	119.0 ± 19.3	141.0 ± 4.6
Histamine - 1.0	113.3 ± 21.8	121.7 ± 3.7 <sup>b</sup>	113.3 ± 6.9	152.7 ± 17.3
- 2.0	167.3 ± 16.7	153.7 ± 23.2	138.0 ± 6.0	174.0 ± 21.4

<sup>a</sup>Values are mean + SEM for three beagles. Values are the maximum percentage response compared to the pre-injection baseline for each dosage of vasoactive compound.

<sup>b</sup>Vasoactive compound caused significant change from baseline (paired  $t$  test, 5% level of significance).

<sup>c</sup>Maximum percentage response post WR 177,602·HCl or WR 142,490·HCl infusion is significantly different from control responses (student  $t$  test, 5% level of significance).

Table 18  
Cumulative Effect of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H on the Cardiorespiratory Responses of Three Beagles<sup>a,b</sup>

Dose of WR 172,435·CH <sub>3</sub> SO <sub>3</sub> H	Mean Arterial Pressure (mmHg)		Heart Rate (bpm)		Respiration (#/min)	
	Baseline	After Drug	Baseline	After Drug	Baseline	After Drug
1.0 mg/kg	129.2 + 5.1	130.0 + 5.2	159.7 + 12.8	158.5 + 13.2	11.2 + 1.2	12.0 + 1.3
3.0 mg/kg	133.3 + 4.6	130.8 + 4.0	155.0 + 8.9	159.2 + 9.2	10.8 + 1.2	12.0 + 1.7
10.0 mg/kg	132.5 + 4.6	106.7 <sup>e</sup> + 8.5	141.0 + 6.7	160.3 + 13.8	10.8 + 1.5	11.2 + 1.2
30.0 mg/kg	132.5 + 4.4	88.3 <sup>e</sup> + 9.1	122.2 <sup>f</sup> + 4.1	137.7 + 8.3	13.5 + 1.1	19.3 + 2.6
100.0 mg/kg <sup>c</sup>	131.7 + 7.3	50.0 <sup>e</sup> + 10.0	102.3 <sup>f</sup> + 2.9	112.3 + 14.5	15.0 + 1.2	32.0 <sup>e</sup> + 5.6
100.0 mg/kg <sup>d</sup>	65.0	35.0	51	62	25	-

<sup>a</sup>Values are mean  $\pm$  SEM.

<sup>b</sup>Doses were administered as an i.v. bolus at 20 minute intervals. Each dose was administered twice. Baseline readings were made immediately prior to drug injection; After Drug readings were made one minute after drug injection.

<sup>c</sup>Only a single determination was made in each dog for this value because two of the three dogs expired after the first 100 mg/kg dose. The average lethal cumulative dose was 255 mg/kg.

<sup>d</sup>These values represent responses in one dog which received a second 100 mg/kg dose.

<sup>e</sup>Significantly different from appropriate baseline (paired  $t$  test, 5% level of significance).

<sup>f</sup>Significantly different from the 1.0 mg/kg baseline value (paired  $t$  test, 5% level of significance).

Table 19  
 Cardiovascular Responses of Three Beagles to a 60 Minute Infusion of  
 WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H, 30 mg/kg<sup>a</sup>

	Time - Minutes						Observation Period		
	WR 172,435·CH <sub>3</sub> SO <sub>3</sub> H, 30 mg/kg Infusion	0	20	40	60	90	120	150	180
Mean Arterial Pressure (mmHg)	148.3 ± 4.4	145.0 ± 2.9	145.0 ± 5.0	143.7 ± 1.7	146.7 ± 1.7	150.0 ± 5.8	143.7 ± 1.7	136.7 ± 6.0	
Heart Rate (bpm)	178.3 ± 12.1	171.0 ± 18.7	164.0 ± 21.7	156.3 ± 20.2	153.3 ± 17.3	144.3 ± 22.3	140.0 ± 20.3	143.6 ± 19.1	
Respiration (#/min)	10.3 ± 3.2	11.0 ± 3.8	12.0 ± 4.2	12.0 ± 3.6	11.0 ± 4.0	11.7 ± 4.4	10.3 ± 3.3	11.0 ± 3.2	
Hematocrit (vol. %)	41 ± 2	43 <sup>c</sup> ± 2	44 <sup>c</sup> ± 2	43 <sup>c</sup> ± 1	--	45 <sup>c</sup> ± 2	--	43 <sup>c</sup> ± 2	

<sup>a</sup>Values are mean ± SEM.

<sup>b</sup>Significantly different from 0 time reading at 5% level of significance, paired t test of significance.

<sup>c</sup>A reddish tint in the plasma was observed in all three animals.

Table 20  
 Cardiorespiratory Responses in Two Beagles to a 60 Minute Infusion  
 of the Vehicle for WR 172,435·CH<sub>3</sub>S0<sub>3</sub>H and WR 194,965·H<sub>3</sub>P0<sub>4</sub><sup>a</sup>

	Infusion of Vehicle			Time - Minutes			Observation Period	
	0	20	40	60	90	120	150	180
Mean Arterial Pressure (mmHg)	184	184	184	185	182	188	182	188
Heart Rate (bpm)	157	160	157	155	158	160	158	155
Respiration (#/min)	7	7	8	8	8	8	9	9
Hematocrit (vol. %)	42	44	44	44	-	44	-	45

<sup>a</sup>Values are the mean of two observations.

Table 21

Mean Arterial Pressure Changes of Three Beagles to Vasoactive Compounds Both before and after a 60 Minute Intravenous Infusion of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H, 30 mg/kg<sup>a,b</sup>

Vasoactive Compound <sup>c</sup>	Responses Before WR 172,435·CH <sub>3</sub> SO <sub>3</sub> H		
	<u>Baseline</u>	<u>Post-Injection</u>	<u>% Baseline</u>
Epinephrine - 2 µg/kg	100.0	121.3	121.3
Isoproterenol - 1 µg/kg	101.3	65.0	64.3
Serotonin - 10 µg/kg	102.0	105.0	103.0
Acetylcholine - 1 µg/kg	101.3	63.3	62.3
Histamine - 2 µg/kg	101.3	67.7	66.3
Angiotensin - 1 µg/kg	100.7	150.0	149.7
Norepinephrine - 2 µg/kg	105.7	135.7	128.3

	Responses After WR 172,435·CH <sub>3</sub> SO <sub>3</sub> H		
	<u>Baseline</u>	<u>Post-Injection</u>	<u>% Baseline</u>
Epinephrine - 2 µg/kg	99.7	121.7	121.7
Isoproterenol - 1 µg/kg	104.0	73.3	69.7
Serotonin - 10 µg/kg	100.3	109.0	108.6
Acetylcholine - 1 µg/kg	100.7	70.7	69.7
Histamine - 2 µg/kg	99.7	70.7	71.0
Angiotensin - 1 µg/kg	107.4	143.3	142.7
Norepinephrine - 2 µg/kg	101.3	129.0	127.7

<sup>a</sup>Values are mean of 3 observations expressed in mmHg.

<sup>b</sup>Drugs administered as a rapid i.v. injection 10 minutes apart. Baseline responses were recorded immediately prior to injection. Post-injection responses were recorded during the peak effect.

<sup>c</sup>Doses were administered as the salts which are listed in the text.

Table 22  
Heart Rate Response of Three Beagles to Vasoactive Compounds Both before  
and after a 60 Minute Intravenous Infusion of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H, 30 mg/kg<sup>a,b</sup>

Vasoactive Compound <sup>c</sup>	Responses Before WR 172,435·CH <sub>3</sub> SO <sub>3</sub> H		
	Baseline	Post-Injection	% Baseline
Epinephrine - 2 µg/kg	155.3	143.3	92.3
Isoproterenol - 1 µg/kg	159.3	216.7	135.7
Serotonin - 10 µg/kg	161.7	146.7	90.0
Acetylcholine - 1 µg/kg	161.7	189.7	117.0
Histamine - 2 µg/kg	161.7	170.0	106.3
Angiotensin - 1 µg/kg	149.7	156.7	104.7
Norepinephrine - 2 µg/kg	146.7	121.7	83.3
Responses After WR 172,435·CH <sub>3</sub> SO <sub>3</sub> H			
Baseline	Post-Injection	% Baseline	
Epinephrine - 2 µg/kg	132.7	143.3	107.3
Isoproterenol - 1 µg/kg	131.0	188.3	143.7
Serotonin - 10 µg/kg	132.7	120.0	90.3
Acetylcholine - 1 µg/kg	135.0	157.0	106.0
Histamine - 2 µg/kg	134.3	140.7	104.0
Angiotensin - 1 µg/kg	132.7	138.7	103.3
Norepinephrine - 2 µg/kg	130.0	122.3	94.3

<sup>a</sup>Values are mean of 3 observations expressed in beats per minute.

<sup>b</sup>Drugs administered as a rapid i.v. injection 10 minutes apart. Baseline responses were recorded immediately prior to injection. Post-injection responses were recorded during the peak effect.

<sup>c</sup>Doses were administered as the salts which are listed in the text.

Table 23

Respiratory Responses of Three Beagles to Vasoactive Compounds Both before  
and after a 60 Minute Intravenous Infusion of WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H, 30 mg/kg<sup>a,b</sup>

<u>Vasoactive Compound<sup>c</sup></u>	<u>Responses Before WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H</u>		
	<u>Baseline</u>	<u>Post-Injection</u>	<u>% Baseline</u>
Epinephrine - 2 µg/kg	8.0	10.7	133.8
Isoproterenol - 1 µg/kg	10.3	19.3	187.4
Serotonin - 10 µg/kg	12.3	18.3	148.8
Acetylcholine - 1 µg/kg	10.0	11.3	113.0
Histamine - 2 µg/kg	9.3	11.0	118.3
Angiotensin - 1 µg/kg	8.3	14.7	177.1
Norepinephrine - 2 µg/kg	7.7	6.7	88.2
<u>Responses After WR 172,435·CH<sub>3</sub>SO<sub>3</sub>H</u>			
<u>Baseline</u>	<u>Post-Injection</u>		
			<u>% Baseline</u>
Epinephrine - 2 µg/kg	9.3	11.3	121.5
Isoproterenol - 1 µg/kg	10.7	17.3	161.7
Serotonin - 10 µg/kg	10.7	18.0	168.2
Acetylcholine - 1 µg/kg	10.0	10.7	107.0
Histamine - 2 µg/kg	10.3	13.3	129.1
Angiotensin - 1 µg/kg	9.7	11.3	116.5
Norepinephrine - 2 µg/kg	9.0	10.7	118.9

<sup>a</sup>Values are mean of 3 observations expressed in #/minute.

<sup>b</sup>Drugs administered as a rapid i.v. injection 10 minutes apart. Baseline responses were recorded immediately prior to injection. Post-injection responses were recorded during the peak effect.

<sup>c</sup>Doses were administered as the salts which are listed in the text.

Table 24  
Cumulative Effect of WR 194,965·H<sub>3</sub>PO<sub>4</sub> on the Cardiorespiratory  
Responses of Three Beagles<sup>a</sup>

Dose of WR 194,965·H <sub>3</sub> PO <sub>4</sub> <sup>b</sup>	Mean Arterial Pressure (mmHg)		Heart Rate (bpm)		Respiration (#/min)	
	Baseline	After Drug	Baseline	After Drug	Baseline	After Drug
1.0 mg/kg	118.7 ± 4.5	116.5 ± 5.2	167.8 ± 9.3	171.3 ± 7.3	7.7 ± 0.7	7.7 ± 0.8
3.0 mg/kg	118.7 ± 1.9	87.7 <sup>d</sup> ± 3.9	152.5 ± 6.6	186.0 <sup>d</sup> ± 4.8	7.3 ± 0.7	8.8 ± 1.3
10.0 mg/kg	114.3 ± 2.2	58.2 <sup>d</sup> ± 4.9	128.3 <sup>e</sup> ± 1.1	168.7 <sup>d</sup> ± 8.4	7.0 ± 0.4	19.3 <sup>d</sup> ± 2.8
30.0 mg/kg <sup>c</sup>	107.0 ± 5.8	35.0 <sup>d</sup> ± 1.8	120.0 <sup>e</sup> ± 2.1	128.8 ± 10.5	6.5 ± 0.3	25.8 <sup>d</sup> ± 3.2

<sup>a</sup>Values are mean ± SEM.

<sup>b</sup>Doses were administered as an i.v. bolus at 20 minute intervals. Each dose was administered twice. Baseline readings were made immediately prior to drug injections. After Drug readings were made one minute after drug injection.

<sup>c</sup>Two dogs expired within 5 minutes of the first injection of 30.0 mg/kg. The third dog expired within 5 minutes of the second 30 mg/kg injection. The average lethal cumulative dose was 68 mg/kg of the salt.

<sup>d</sup>Significantly different from appropriate baseline (paired *t* test, 5% level of significance).

<sup>e</sup>Significantly different from 1.0 mg/kg baseline (paired *t* test, 5% level of significance).

Table 25  
Cardiorespiratory Responses of Three Beagles to a 60 Minute  
Infusion of WR 194,965·H<sub>3</sub>PO<sub>4</sub>, 30 mg/kg Base<sup>a</sup>

	WR 194,965·H <sub>3</sub> PO <sub>4</sub> , 30 mg/kg Base Infusion						Observation Period	
	0	20	40	60	90	120	150	180
Mean Arterial Pressure (mmHg)	136.7 + 9.3	140.0 + 7.7	133.0 + 5.1	130.7 + 2.3	126.7 + 1.7	128.3 + 3.5	122.7 + 6.2	127.3 + 6.2
Heart Rate (bpm)	177.7 + 12.5	147.3 <sup>b</sup> + 14.5	130.7 <sup>b</sup> + 13.3	126.0 <sup>b</sup> + 8.6	123.0 <sup>b</sup> + 10.1	122 <sup>b</sup> + 11.6	118.0 <sup>b</sup> + 9.7	120.0 <sup>b</sup> + 5.0
Respiratory Rate (#/min)	12.7 + 2.6	12.7 + 3.3	10.3 + 2.7	8.7 + 2.3	7.7 + 1.5	8.0 + 2.1	9.0 + 2.3	8.3 + 2.0
Hematocrit (vol. %)	40 + 1	41 <sup>c</sup> + 2	42 <sup>c</sup> + 2	42 <sup>c</sup> + 2	--	39 <sup>c</sup> + 1	--	38 <sup>c</sup> + 1

<sup>a</sup> Mean + SEM for 3 animals.

<sup>b</sup> Significantly different from 0 time reading at 5% level of significance, paired t test of significance.

<sup>c</sup> A reddish tint in the plasma was observed in all 3 animals.

Table 26

Cardiorespiratory Responses of Three Beagles to a 40 Minute Infusion of WR 194,965·H<sub>3</sub>PO<sub>4</sub>, 15 mg/kg Base<sup>a,b</sup>

	Time - Minutes				
	WR 194,965·H <sub>3</sub> PO <sub>4</sub> , 15 mg/kg Base Infusion	0	10	20	30
Mean Arterial Pressure (mmHg)	123.3 + 14.5	122.3 + 14.1	120.3 + 13.0	117.0 + 16.3	118.3 + 19.2
Heart Rate (bpm)	163.7 + 3.3	152.3 + 4.7	145.7 + 8.1	136.0 + 10.7	126.7 <sup>c</sup> + 9.3
Respiration (#/min)	9.0 + 3.1	8.3 + 3.0	7.3 + 2.9	6.7 + 2.7	5.7 + 2.7

<sup>a</sup>These beagles were those subjected to the series of vasoactive agents prior to and after the infusion.

<sup>b</sup>Values are mean  $\pm$  SEM.

<sup>c</sup>Significantly different from 0 time reading at 5% level of significance, paired  $t$  test of significance.

Table 27

Mean Arterial Pressure Changes of Three Beagles to Vasoactive Compounds Both before  
and after a 40 Minute Infusion of WR 194,965-H<sub>3</sub>PO<sub>4</sub>, 15 mg/kg Base<sup>a,b</sup>

<u>Vasoactive Compound</u>	Responses Before WR 194,965-H <sub>3</sub> PO <sub>4</sub>		
	<u>Baseline</u>	<u>Post-Injection</u>	<u>% Baseline</u>
Epinephrine - 2 µg/kg	128.3	183.0	144.3
Isoproterenol - 1 µg/kg	124.3	64.3	54.3
Serotonin - 10 µg/kg	121.7	119.3	98.0
Acetylcholine - 1 µg/kg	134.3	78.3	59.3
Histamine - 2 µg/kg	126.0	85.0	67.3
Angiotensin - 1 µg/kg	129.3	216.7	170.0
Norepinephrine - 2 µg/kg	124.3	166.0	132.3

<u>Vasoactive Compound</u>	Responses After WR 194,965-H <sub>3</sub> PO <sub>4</sub>		
	<u>Baseline</u>	<u>Post-Injection</u>	<u>% Baseline</u>
Epinephrine - 2 µg/kg	120.0	185.0	160.0
Isoproterenol - 1 µg/kg	123.3	74.0	61.3
Serotonin - 10 µg/kg	122.0	114.3	93.7
Acetylcholine - 1 µg/kg	129.3	80.7	63.0
Histamine - 2 µg/kg	128.3	80.7	63.0
Angiotensin - 1 µg/kg	118.7	228.7	202.7
Norepinephrine - 2 µg/kg	126.7	171.7	136.0

<sup>a</sup>Values are mean of 3 observations expressed in mmHg.

<sup>b</sup>Drugs administered as a rapid i.v. injection 10 minutes apart. Baseline responses were recorded immediately prior to injection. Post-injection responses were recorded during the peak effect.

<sup>c</sup>Doses were administered as the salts which are listed in the text.

Table 28

Heart Rate Responses of Three Beagles to Vasoactive Compounds Both before  
and after a 40 Minute Infusion of WR 194,965·H<sub>3</sub>PO<sub>4</sub>, 15 mg/kg Base<sup>a,b</sup>

Vasoactive Compound <sup>c</sup>	Responses Before WR 194,965·H <sub>3</sub> PO <sub>4</sub>		
	Baseline	Post-Injection	% Baseline
Epinephrine - 2 µg/kg	160.3	181.0	114.0
Isoproterenol - 1 µg/kg	166.7	215.7	129.7
Serotonin - 10 µg/kg	163.7	152.7	93.3
Acetylcholine - 1 µg/kg	167.0	176.0	105.0
Histamine - 2 µg/kg	167.0	166.7	99.7
Angiotensin - 1 µg/kg	167.0	201.7	120.7
Norepinephrine - 2 µg/kg	163.7	177.7	108.3
Responses After WR 194,965·H <sub>3</sub> PO <sub>4</sub>			
Baseline	Post-Injection		% Baseline
Epinephrine - 2 µg/kg	131.3	158.0	120.3
Isoproterenol - 1 µg/kg	131.7	186.0	142.3
Serotonin - 10 µg/kg	135.3	128.7	95.0
Acetylcholine - 1 µg/kg	136.0	142.3	104.3
Histamine - 2 µg/kg	133.7	139.3	103.7
Angiotensin - 1 µg/kg	129.0	156.3	121.7
Norepinephrine - 2 µg/kg	137.7	167.3	120.7

<sup>a</sup>Values are a mean of 3 observations expressed in beats per minute.

<sup>b</sup>Drugs administered as a rapid i.v. injection 10 minutes apart. Baseline responses were recorded immediately prior to injection. Post-injection responses were recorded during the peak effect.

<sup>c</sup>Doses were administered as the salts which are listed in the text.

Table 29

Respiratory Responses of Three Beagles to Vasoactive Compounds Both before  
and after a 40 Minute Infusion of WR 194,965·H<sub>3</sub>P0<sub>4</sub>, 15 mg/kg Base<sup>a,b</sup>

Vasoactive Compound <sup>c</sup>	Responses Before WR 194,965·H <sub>3</sub> P0 <sub>4</sub>		
	Baseline	Post-Injection	% Baseline
Epinephrine - 2 µg/kg	8.0	8.3	103.8
Isoproterenol - 1 µg/kg	8.0	12.0	150.0
Serotonin - 10 µg/kg	8.7	14.3	164.5
Acetylcholine - 1 µg/kg	8.7	9.7	111.5
Histamine - 2 µg/kg	8.0	9.0	112.5
Angiotensin - 1 µg/kg	8.0	9.7	121.3
Norepinephrine - 2 µg/kg	8.7	9.3	106.9
Responses After WR 194,965·H <sub>3</sub> P0 <sub>4</sub>			
Baseline	Post-Injection	% Baseline	
Epinephrine - 2 µg/kg	5.8	6.7	115.5
Isoproterenol - 1 µg/kg	6.7	12.0	179.1
Serotonin - 10 µg/kg	5.7	10.3	180.7
Acetylcholine - 1 µg/kg	5.7	6.3	110.5
Histamine - 2 µg/kg	6.7	8.0	119.4
Angiotensin - 1 µg/kg	5.7	7.3	128.1
Norepinephrine - 2 µg/kg	6.7	7.6	113.4

<sup>a</sup>Values are a mean of 3 observations expressed as #/minute.

<sup>b</sup>Drugs administered as a rapid i.v. injection 10 minutes apart. Baseline responses were recorded immediately prior to injection. Post-injection responses were recorded during the peak effect.

<sup>c</sup>Doses were administered as the salts which are listed in the text.

Table 30  
Analysis of "Unknown" Samples of WR 30,090

Tube #	Actual Value ( $\mu\text{g}/\text{ml}$ )	Recovery by Means of Analytical Procedure	% Difference
1	1.651	1.596	3.3
2	1.396	1.401	0.4
3	0.192	0.194	1.0
4	0.283	0.300	6.0
5	0.676	0.672	3.0
6	1.221	1.277	4.6
7	0.676	0.620	5.0
8	BROKEN -----		
9	0.283	0.294	3.9
10	1.395	1.397	0.1
11	1.221	1.253	2.6
12	1.651	1.574	4.7

Project 3M762770A803 MALARIA PROPHYLAXIS

Task 00 Malaria Investigations

Work Unit 087 Determination of pharmacological effects of anti-malarial drugs

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>2</sup>	2. DATE OF SUMMARY <sup>3</sup>	REPORT CONTROL SYMBOL
3. DATE PREV SUMMARY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY <sup>4</sup> U	6. WORK SECURITY <sup>5</sup> U	DA OC 6446	77 10 01	DD-DR&E(A-R)636
7. REGRADING	8. DOD/IN INSTN <sup>6</sup> NA	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT			
10. NO./CODES: <sup>7</sup> b. PRIMARY 62770A	PROGRAM ELEMENT PROJECT NUMBER 3M762770A803	11. WORK UNIT NUMBER 00	12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>8</sup> 002300 Biochemistry 012900 Physiology			
c. CONTRIBUTING CARDS 114 F			13. START DATE 76 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House
d. CONTRACT/GRANT e. DATES/EFFECTIVE: NA	EXPIRATION:	17. RESOURCES ESTIMATE FISCAL YEAR 77 CURRENcy	18. PROFESSIONAL MAN YRS 8	19. FUND (In thousands) 656		
f. NUMBER: g. TYPE: h. KIND OF AWARD:	4. AMOUNT: f. CUM. AMT.	20. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Division of Biochemistry Washington, DC 20012	21. GENERAL USE Foreign Intelligence not considered	PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME: DOCTOR, B.P. Ph.D TELEPHONE: (202) 576-3001 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]	ASSOCIATE INVESTIGATORS NAME: SLEEMAN, H. KENNETH Ph.D NAME: KAZYAK, LEO	DA
22. KEYWORDS (Precede EACH with Security Classification Code)				(U) Antimalarials (U) Pharmacokinetics (U) Analytical Chemistry (U) Drug Intolerance		
23. TECHNICAL OBJECTIVE, <sup>9</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede rest of each with Security Classification Code.)						
<p>23. (U) The technical objectives include (1) the determination of biochemical indices that reliably predict drug intolerance in man. (2) The evaluation of effects of parasites and drugs on biochemical mechanism as indices of prophylaxis and treatment. (3) The study of antigen variability in trypanosomes which enables the organism to elude the immune response of the host. (4) Analytical support for special and collaborative projects. The prediction of drug intolerance, the effects of biochemical mechanisms and the understanding of antigenic variations in trypanosomes are relevant to the Army's malaria and tropical disease program.</p> <p>24. (U) Tissues and fluids from animals will be analyzed to determine metabolic processes affected by parasitic diseases and therapeutic and prophylactic drugs. Metabolic products enzymes, isoenzymes, nucleic acids and proteins will be the primary focus. Immunochemical, spectrophotometric, mass spectrometric and chromatographic methods will be developed and utilized to analyze biological specimens for compounds of interest.</p> <p>25. (U) 76 10 - 77 09 Studies have continued on the effects of antimalarials on physiological and metabolic parameters. Nucleotide profiles of rhesus monkey basal, during infection and after treatment have been determined. Gas chromatographic-coupled mass spectrometric analyses of antimalarials and metabolites have been continued. Trypanosomiasis infections are established in the rat and studies on metabolism and antigenic variation have begun. Assay of drugs for antifolate activity is continuing. For technical reports see WRAIR Annual progress report 1 Jul 76 to 30 Sep 77.</p>						
1363						

\*Available to contractors upon originator's approval.

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Project 3M76770A803 MALARIA PROPHYLAXIS AND TREATMENT

Task Malaria Investigation

Work Unit 088 Biochemical Research in Antimalarials

Investigators.

Principal: Bhupendra P. Doctor, Ph.D.

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Craig G. Zempel, B.S.

The effort under this work unit involved certain aspects of the biochemistry of malaria, biochemical effects of antimalarials, the development of methods for the analysis of experimental antimalarials, and the speciation of biomolecules by mass spectrometry. Initial studies on the development of a model for the study of Trypanosoma rhodesiense also are reported.

1. Blood nucleotide profiles produced by malaria infection and chloroquine prophylaxis or treatment.
  2. Analysis of Mefloquine (WR142490) and 2,4-Diamino-6-(2-naphthylsulfonyl) quinazoline (WR158122).
  3. Biochemical effects of antimalarials.
  4. Methodology for the study of antifolates.
  5. Studies on experimental African trypanosomiasis
  6. Phthalate plasticizers in biological fluids.
  7. Evaluation of results on the metabolism of methaqualone.
  8. Evaluation of the Lockheed Dialog information retrieval system.
1. Blood nucleotide profiles produced by malaria infection and chloroquine prophylaxis or treatment.

This was a collaborative study with the Department of Hematology, Division of Medicine, and Department of Experimental Pathology, Division of Pathology. Refer to the Annual Reports from these Departments for additional information on this study.

Changes in the content and distribution of blood nucleotides (NTC) were studied in rhesus monkey under three experimental conditions: synchronous infection with *P. knowlesi*, chloroquine prophylaxis, and chloroquine treatment of fulminant malaria infection. Perchloric acid extracts of whole blood after neutralization were analyzed using a high performance liquid chromatographic system with a microparticle anion exchange column packing and a  $K_2HPO_4$  linear gradient (0-100%). Dramatic changes were observed in adenylylates, although variations were recorded for all major ribonucleotides. Infected monkeys followed over 2-3 cycles showed variation in NTD pools characteristic of the predominant schizogonic growth stage. During "ring" stage, ATP levels, total adenylylates, and adenylylate energy charge were maintained at or above basal values independent of parasitemia. During trophozoite stage and schizogony, the above parameters were decreased markedly. Uninfected monkeys given therapeutic doses of chloroquine showed dose/time dependent decreases in ATP levels and adenylylate energy charge, whereas total adenylylates decreased slightly. Return toward basal levels correlated with the biological half-life of chloroquine in monkeys. In chloroquine-treated infected monkeys, ATP levels decreased, adenylylate energy charge stabilized, and stage-related variations in the these parameter did not occur. Studies on nucleotide levels in malaria before and after treatment are continuing.

## 2. Analysis of Mefloquine (WRI42490) and WR 158122.

A method utilizing  $Ni^{63}$  electron capture gas chromatography to detect mefloquine concentrations as low as 20 ng/ml in plasma has been developed. The method is adequate for most determinations of mefloquine in blood or plasma. The use of WR 184,806 as an internal standard provides an effective means of maintaining precision of the method.

Preliminary studies involving the use of GC-mass spectrometry indicated that mefloquine could be detected in amounts as little as 5ng. (on column) without difficulty. However, this sensitivity is based on utilizing m/e 84 (base peak) piperidyl fragment in the Electron Impact (EI) mode for select ion monitoring. Specificity is not so good as in monitoring the molecular ion, but the relative intensity of the molecular ion is much too low in the E.I. mode to be useful. Even in the Chemical Ionization mode, the  $M+1$  ion fragment has a relative intensity that is too low to detect nanogram concentrations

Since the methodology for mefloquine determination is considered adequate for analytical support of clinical studies, no further work is being conducted on this compound.

Efforts to develop an effective gas chromatographic procedure for 2,4-diamino-6-2(2-naphthylsulfonyl) quinazoline (WR 158,122) have been unsuccessful thus far, because the compound does not appear to volatilize sufficiently for this purpose, and no derivatives have been found that would overcome the difficulty. Attempts to produce a suitable derivative will continue.

### 3. Biochemical effects of antimalarials.

Studies on the biochemical effects of antimalarials are continuing after a substantial delay caused by reorganization of the Division of Biochemistry. The depression of serum T<sub>4</sub> levels produced by WR 142490 (WRAIR Progress Report FY 75-76) was confirmed. Also, the administration at doses of 125 mg/kilogram body weight of another quinoline methanol, WR 30090, produced a similar decrease in serum T<sub>4</sub> levels. Studies on the effects of the quinoline methanols on thyroid function and energy production have been initiated.

### 4. Methodology for the study of antifolates:

#### Interaction of drugs with Lactobacillus casei dihydrofolate reductase.

The competitive protein binding assay based on the tight binding of drugs to L. casei dihydrofolate reductase has a broad application to antifolate research. The technique allows the direct measurement of the association constants of antifolates and dihydrofolate reductase under optimum binding conditions. Studies were initiated to establish an antifolate screen for drugs prepared for WRAIR. Assays for WR 38839, WR 99210, WR5949, and WR 159412 were attempted. Data obtained from studies on these drugs were not conclusive possibly because of their limited solubility in aqueous systems or because of their strong affinity for protein. Work on improving the methodology is continuing.

#### Methotrexate detection in WRAMC Patients Undergoing High Dose Therapy.

These studies were undertaken in collaboration with Drs. Raryman and Mease, Department of Pediatrics, and Drs. Blum, Babcock, Miller, and San Antonio, Hematology Oncology Clinic, WRAMC.

Methotrexate, an inhibitor of dihydrofolate reductase, is widely used as an antineoplastic and immuno suppressive agent. Its use both by systemic and intrathecal regimens has been hampered by occasional toxicity. Therefore, routine monitoring of blood levels of Methotrexate in patients under therapy is necessary. A competitive protein binding assay (Myer et al., 1975) has been modified to determine the blood levels of methotrexate in patients. The assay has been set up and evaluated, and over 110 clinical samples have been analyzed to date. The methodology will be transferred eventually to the clinical laboratory at WRAMC.

##### 5. Studies on Experimental African Trypanosomiasis

Under the influence of several regulatory hormones, the formation of glucose and its release into the blood involve either: (1) release from preformed glycogen; (2) recycling of glucose-derived intermediates, such as lactate, pyruvate and glycerol; or (3) de novo glucose synthesis from precursor amino acids. Recent studies exploring the mechanisms of terminal hypoglycemia in experimental African trypanosomiases suggest that, in addition to diminished stores of hepatic glycogen, reduced endogenous production of glucose by gluconeogenesis may also contribute to the development of hypoglycemia.

Some of the mechanisms which contribute to inadequate gluconeogenesis may include enzymatic inhibition or defect, decreased hormonal regulation, or substrate deficiency.

The present study was undertaken to provide comparative data on gluconeogenesis in both rats and guinea pigs infected with Trypanosoma rhodesiense. In rats, the trypanosomes multiply, rapidly killing the host in several days; in guinea pigs the infection assumes a more chronic form, but ultimately causes death.

The initial phase of this study involved the standardization of infections in experimental host. A subsequent phase, now in progress, deals with the levels of activity of 4 obligatory gluconeogenic enzymes (glucose-6-phosphatase, fructose-1,6-diphosphatase, phosphoenolpyruvate carboxy kinase and pyruvate carboxylase) in infected livers, as compared to non-infected controls. Other aspects of this study will deal with possible alterations in hormonal regulation of gluconeogenesis, as well as with available substrate or precursor levels.

## 6. Phthalate plasticizers in biological fluids

For the past ten years concern has been growing over the fact that chemical plasticizers have been contaminating biological systems. The most notable example is the amount of diethylhexyl phthalate (DEHP) introduced into the human body during administration of blood that has been stored in plastic containers. Toxicity studies on di(2-ethylhexyl) phthalate show its toxicity to be so low that the compound can be regarded as non-toxic. It was established that DEHP disappeared rapidly from the blood concomitant with a high clearance rate in the kidneys and urine; little or no phthalic acid was produced as a by-product of metabolism. However, evidence from recent studies by Albro and Moore points to the formation of mono-ethylhexyl phthalate metabolite that has some toxicity. This information caused new concerns in the field of organ transplantation where any potential for toxicity is increased, especially for kidney transplants where renal clearance is impaired. Hence, the present study is centered on the fate and metabolism of DEHP as it is incorporated into blood that passes through polyvinyl chloride tubing which contains large amounts of DEHP plasticizer. This is a collaborative study with WRAMC in order to provide GS-MS expertise.

During the two months that this study has been in progress, a method has been developed that utilizes the gas chromatograph mass spectrometer technique of select ion monitoring. Specimens of blood drawn from patients during kidney dialysis have been employed to follow the level of the plasticizers. To date, the levels in patients studied have been very low with very little ostensible change in the level of phthalate during the dialysis period. The fact that phthalate is consistently detected in the blood samples has prompted a more comprehensive analysis for intermediates and metabolites of DEHP. Some preliminary observations monitored by mass spectrometry have shown the spectral characteristics very similar to metabolites of DEHP that have been reported by Alboro and Fishbein after feeding DEHP to rats and analyzing the urine. Since no urine is produced by the patients studied, the possibility of reabsorption of DEHP metabolites presents a serious threat to any successful organ transplantation if these metabolites are as toxic as suspected.

To implement the metabolite studies, liver perfusion experiments are being conducted with pigs (at NIH). Expired blood, stored in plastic containers and having a high DEHP concentration, is infused. The liver is isolated by ligating the superior vena cava and the blood is circulated through cannulas in the portal vein and inferior vena cava. Presumably

metabolites formed during this perfusion will concentrate in blood, and thereby provide a useful model for following biotransformations of DEHP, as well as a source of metabolites for comparison with the analyses of blood from patients.

Although DEHP toxicity is a continuing problem associated with storage of biological fluids in plastic containers, the immediate impact we have encountered in our investigation concerns its effect on mixed lymphocyte reactions. Recently it has been shown that many of the observations made using blood lymphocytes from the patients who are on dialysis treatment were contrary to expected results. Our investigation appears to relate this discrepancy in results to toxicity of DEHP in MLR.

#### 7. Evaluation of results on the metabolism of methaqualone

The following resume constitutes the final evaluation of results of the methaqualone study in conjunction with drug abuse research (WRAIR Progress Report FY 75-76).

Five principal urinary metabolites of methaqualone were measured in seven volunteers following single and multiple doses of the drug. Urine collected for up to 72 hours after the last dose was analyzed for methaqualone and its metabolites by high resolution capillary column gas chromatography. The major biotransformation of methaqualone under therapeutic conditions occurred through benzylic and para-hydroxylation of the O-tolyl moiety. Methaqualone itself was present in concentrations of no more than 1ug/ml if it could be detected at all. The total urinary excretion of metabolites reflected the cumulative nature of the parent drug when administered in multiple dose. No clear relationship was found between appearance of a specific metabolite and time elapsed after ingestion of the drug, although higher amounts of 2-methyl-3-(2'-hydroxymethylphenyl)-4 (3H) quinazolinone (I) were noted in individuals with low tolerance to the drug.

The majority of urinary methaqualone metabolites have been reported to be bound as glucuronide conjugates. Since it was also known that hydrolysis with a mineral acid destroys much of the sample, an enzymatic hydrolysis with glusulase was employed to cleave any conjugates under mild conditions. Experiences with acid hydrolysis indicated that metabolite II (2-hydroxy methyl-3-O-tolyl-4 (3H)-quinazolinone) was particularly susceptible to acid degradation and indeed to enzymatic hydrolysis. Enzymatic hydrolysis is not without artifacts, however, since Stillwell *et al.* have suggested that a metabolite is formed by dehydration rearrangement of a dihydrodiol during incubation with glusulase.

The isomeric metabolites 2-methyl-3-(2-methyl-3-hydroxy phenyl)-4(3H)-quinazolinone (III), 2-methyl-3-(2-methyl-4-hydroxy phenyl)-4(3H)-quinazolinone (IV), and 2-methyl-3-o-tolyl -6 hydroxy-4(3H)-quinazolinone (V) were not completely resolved on packed columns containing either non-polar (SE-30) or moderately polar (OV-17, OV-210) liquid phases. The separation of these metabolites as their trimethylsilyl derivatives with sufficient resolution for quantification was possible with a high resolution SCOT capillary column. The high capacity and stability of this column allowed use of a splitless injection technique for increased sensitivity and precision.

A packed column that could accommodate a larger sample injection volume was initially used for methaqualone determinations, since the urinary concentrations of the parent drug were consistently too low to be detected in 1.0  $\mu$ l volume of sample normally injected on the capillary column. However, the packed column was unable to separate methaqualone completely from extraneous substances, and the resulting matrix effects impaired quantitative accuracy. Selected ion monitoring with a GC-MS system eventually solved the problem, but not before many of the samples had been depleted. As well as could be estimated, the highest concentration of methaqualone for a therapeutic dose of 300 mg was no more than 1.0  $\mu$ g/ml urine. Over half of the urines (58.5%) that showed any evidence of methaqualone contained less than 50  $\mu$ g of the unchanged drug in the total collection, and at least half of these specimens were estimated at no more than 10-20  $\mu$ g in the total volume of a casual urine collection.

Of the five major metabolites measured, I and IV exceeded the amounts of the others. Therefore, hydrosylation of methaqualone tends to occur predominantly on the O-tolyl ring at the 4 position, which is the most susceptible locus for electrophilic substitution, or at the reactive benzylic carbon. The relationship of these two metabolites is not clear, but it is noteworthy that where metabolite I is predominant, the effects of the drug on the individual were more pronounced, and those persons were less tolerant of the drug than the others who manifested greater concentrations, overall, of metabolite IV. Two of the participants on multiple dose schedules had more intense effects after the final dose, and it is interesting to note that the concentrations of metabolites I and IV at that point were approximately the same. Bonnichsen reported that metabolite I was present unconjugated, together with methaqualone in blood from cases of intoxication by metabolite I and its relation to acute intoxication. Unfortunately, our data from therapeutic doses cannot resolve these questions, but the pattern is consistent for the few examples that have been studied. At the very least, this compound, which is

synthetically available, should be examined for sedative and hypnotic activity as well as toxicity. It may well be that this metabolite is active in the same as, or in a different manner from, the parent drug.

Attempts to draw quantitative comparisons with the findings of others were difficult, because they do not specify the total amounts of metabolite. Bonnichsen reported quantities either in ug/ml or relative percentages of total metabolite, as determined by ultraviolet spectrophotometry; hence, some of the data included unchanged methaqualone as well as metabolite. Some of the metabolite concentrations determined in our study, when expressed in ug/ml, were higher than those reported by Bonnichsen for suicide cases that involved methaqualone overdose. Values for metabolite I were as high as 70-90 ug/ml for a collected volume of 100-200 ml of urine within 8-12 hours after the dose. In our experience metabolite levels expressed as ug/ml (mg%) tended to fluctuate greatly from one specimen to another, and some of the fluctuation was due to differences in the volume of urine excreted.

The irregular amounts of metabolite excreted confirm the findings of Reavey *et al.* and leave the interpretation of single determinations open to question. Our study has focused on the five principal metabolites that could be identified and quantified. Other investigators have reported at least five additional metabolites that might be important in establishing links between a state of overdose and ordinary manifestations of therapeutic levels of the metabolites. Information derived from one dose regimen can only suggest areas for further investigation and establish some basis for comparisons. More quantitative data from cases of intoxication will be needed before the significance of metabolite determinations can be completely evaluated.

#### 8. Evaluation of the Lockheed Dialog information retrieval system.

##### Special Assignment

Dr. Garson was detailed from 21 September 1976 to 20 January 1977 to a special project involving an evaluation, within the WRAIR, of the Lockheed DIALOG computerized on-line information retrieval system. Duties pertained to organization and implementation of the trial evaluation period, which included training of designated search analysts, orientation for personnel of the WRAIR, and analysis of critiques and comments submitted by WRAIR users of the system as a result of a successful trial, the DIALOG information retrieval system has been adopted for use by the WRAIR for an indefinite period of time.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY						I. AGENCY ACCESSION <sup>6</sup> DA OB 64;1	II. DATE OF SUMMARY <sup>6</sup> 77 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMMARY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCRTY <sup>6</sup> U	6. WORK SECURITY <sup>6</sup> U	7. REGRADING <sup>6</sup> NA	8. DISPN INSTRN <sup>6</sup> NL	9. SPECIFIC DATA-CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	10. LEVEL OF SUM A. WORK UNIT	
10. NO./CODES: <sup>6</sup> 62770A	PROGRAM ELEMENT PROJECT NUMBER 3M762770A803			TASK AREA NUMBER 05	WORK UNIT NUMBER 089			
11. PRIMARY B. CONTRIBUTING C. EXCLUDED FROM WORK CARDS 114F								
11. TITLE (Pecede with Security Classification Code) (U) Field Studies on Drug Resistant Malaria								
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>6</sup> 003500 Clinical Medicine 010100 Microbiology								
13. START DATE 69 07	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House				
17. CONTRACT/GANT B. DATES/EFFECTIVE: NA EXPIRATION:			18. RESOURCES ESTIMATE PRECEDING FISCAL YEAR 77	19. PROFESSIONAL MAN YRS CURRENT 6	20. FUND'S (In thousands) 481			
C. NUMBER: G. TYPE: H. KIND OF AWARD:	D. AMOUNT: E. CUM. AMT.			78	7.5	500		
19. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research			20. PERFORMING ORGANIZATION NAME: US Army Medical Component, AFRIMS					
ADDRESS: Washington, DC 20012			ADDRESS: Bangkok, Thailand					
RESPONSIBLE INDIVIDUAL NAME: RAPMUND, G., COL TELEPHONE: 202-576-3551			PRINCIPAL INVESTIGATOR (Pecede with Security Classification Code) NAME: SEGAL, H. E., LTC TELEPHONE: 281-7776 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]					
21. GENERAL USE Foreign intelligence not considered			ASSOCIATE INVESTIGATORS NAME: DOBERSTYN, E. B., LTC NAME: BROWN, J. L., MAJ					
22. KEYWORD (Pecede EACH with Security Classification Code) (U) Malaria; (U) Drug Resistance; (U) Chemotherapy; (U) Human; (U) Monkey; (U) Vectors; (U) Chloroquine; (U) Human Volunteer								
23. TECHNICAL OBJECTIVE, <sup>6</sup> 24. APPROACH, 25. PROGRESS (Pecede individual paragraphs identified by number. Pecede text of each with Security Classification Code.) 23. (U) To determine the effect of conventional and experimental antimalarial drugs in the treatment, prophylaxis, and transmission of drug-resistant falciparum malaria, a disease of continuing military importance. To define vector bionomics which influence the transmission of drug-resistant malaria and to develop rationales for vector control. To evaluate the activity of candidate antimalarial drugs against simian malaria. To characterize the cellular immune response of patients infected with malaria. To characterize the clinical features of malaria in Thailand and to develop improved methods of hospital management. 24. (U) US Army investigational antimalarial drugs were compared with standard drugs for treatment of drug-resistant falciparum malaria in hospitalized human volunteers in Thailand. Lymphocytes from patients infected with drug resistant malaria were isolated and their response to malarial antigen was characterized. Chemotherapeutic drugs were studied in rhesus monkey with Plasmodium cynomolgi. 25. (U) 76 10 - 77 09 WR 142,490 (mefloquine) in a single oral dose was more effective than Fansidar in the treatment of mildly and moderately ill patients with P. falciparum malaria. It cured 100 percent of patients followed for 28 days. Fansidar cured 91 percent. The therapeutic effect of several drug regimens against P. vivax was compared and mefloquine was found to be as effective as chloroquine. Fansidar was ineffective. Studies of the gametocytocidal and sporonticidal effect of mefloquine are underway. The reactivity of leukocytes from malaria patients to stimulation by non-specific agents and malaria antigen has been characterized. The malaria drug testing program has used the rhesus monkey-Plasmodium cynomolgi system to test 107 drugs for schizonticidal and/or radical curative effects. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76-30 Sep 77.								
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**Project 3M762770A803 MALARIA PROPHYLAXIS**

**Work Unit 089 Field studies on drug resistant malaria (SEATO)**

**Investigators.**

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1. The Suppression of Plasmodium falciparum and Plasmodium vivax Parasitemias by Mefloquine (WR142490, A 4-Quinolinemethanol)

**OBJECTIVES:** To study the efficacy of the 4-quinolinemethanol, mefloquine (WR142490), in suppressing parasitemias in an area endemic for multidrug resistant P. falciparum and P. vivax malaria.

To compare the efficacy of mefloquine with that of the combination sulfadoxine-pyrimethamine

**BACKGROUND:** Mefloquine (WR142490) is an a-(piperidyl)-4-quinolinemethanol which appeared to be longer acting than a previously studied parent compound, WR 30090. Following evidence of the lack of toxicity in early studies, together with demonstration of the efficacy of Mefloquine as a chemotherapeutic and chemosuppressive agent, expanded studies, including the one reported here, were approved for conduct in Thai populations.

**METHODS:** The Bu Phram valley area, site of previous drug trials in Prachinburi Province, was again used. At the outset of the study, a census was taken and the villages were surveyed. The study protocol was explained to the villagers and those who wished to participate were asked to sign a consent form. The study population, numbering 1,050, were randomly assigned to one of five major treatment groups and a smaller placebo group (Table 1). Under a double blind design, all participants received two tablets under observation each week. In the case of those on medication every other week or on one tablet each week placebo tablets were given. Younger participants in the study (weighing 22-35 kilograms) were given exactly 1/2 the adult dose of Mefloquine. In the case of sulfadoxine-pyrimethamine no such reduction was deemed necessary. Study subjects were visited weekly by Laboratory technicians, who inquired about the villager's health, and illnesses or fevers during the past week, and whether the study subject had received any medications from an outside source. Every week a physician was available to see any ill study subject. A capillary blood was drawn for a thick-thin malaria slide, white blood cell count and microhematocrit. In the course of the trial, three venapunctures were done for determining biochemical parameters.

**RESULTS:** Nine hundred ninety study subjects began the 26 week field trial and 856 completed it (86.5%). There was no evidence of selection bias between the six treatment groups. Malaria transmission did occur during the field trial, with calculated attack rates of greater than 1000/1000/yr for both P. falciparum and P. vivax. Among 487 individuals receiving Mefloquine, there were only three instances of a falciparum parasitemia (Table 2). By contrast, nine villagers out of 333 receiving S-Py had a total of 12 episodes of falciparum parasitemia. Nineteen study subjects given placebo experienced a total of 40 episodes of parasitemia during the same time period. The duration of an asexual parasitemia

**Table 1. Treatment Groups and Dose Schedule for Study Subjects During Chemosuppression for 26 Weeks**

No. Subjects Starting Study	Dosage Schedule
190	S(1000mg)-Py(50mg) every two weeks
192	S(500mg)-Py(25mg) every week
189	M(180mg) every week, children 22-35 kgs. 1/2 dose
191	M(360mg) every week, children 22-35 kgs. 1/2 dose .
184	M(360mg) every two weeks, children 22-35 kgs. 1/2 dose
44	PLACEBO

Table 2. *P. falciparum* Parasitemias Experienced by Study Subjects  
 During Chemosuppression for 26 Weeks

Group	No. Subjects Completing Study	No. (Prop.) Infected	No. Episodes	Ave. Duration (Weeks) of Episode
S (1000mg)-Py (50mg) fortnightly	162	5 (0.03)	8	2
S (500mg)-Py (25mg) weekly	171	4 (0.02)	4	3
Mefloquine (180mg) weekly	160	1 (0.01)	1	1
Mefloquine (360mg) fortnightly	169	0 (0.00)	0	-
Mefloquine (360mg) weekly	158	2 (0.01)	2	1
Placebo	36	19 (0.53)	40	2

in the S-Py and placebo groups was longer than the Mefloquine groups, although not statistically significantly so. Figure 1 shows the cumulative falciparum infection rates of the groups during the 26 week chemosuppressive phase and in the three month follow-up period. Malaria transmission was generally constant and there was no apparent loss of effectiveness during the study period in any of the drug treatment groups. After the chemosuppressive phase, the number of new falciparum infections in study subjects from the drug treatment groups increased. The three Mefloquine treatment groups had fewer study subjects contracting their first episode of falciparum malaria during the follow-up phase compared with the S-Py groups.

The number of vivax parasitemias among study subjects receiving Mefloquine during chemosuppression was small (4 cases) compared with the S-Py groups (28 cases) (Table 3). During the 26 week period of chemosuppression, there was a continuous accumulation of vivax parasitemias in the placebo group, reaching a proportion of 0.80 at 17 weeks (Figure 2). The rate of accumulation of parasitemias in the S-Py groups appeared to increase in the latter weeks of the study. This was followed by a marked increase in the proportion of individuals with vivax parasitemias occurring after termination of drug suppression in all groups.

Statistical evaluation comparing the various drug groups was undertaken using as the parameters the number of positive species-specific slides and the number of negative slides (Table 4). Evaluation between groups shows that Mefloquine, using the three Mefloquine groups combined, to be more efficacious than either of the S-Py regimens in suppressing a falciparum parasitemia ( $p < 0.001$ ). The Fisher Exact Test fails to reveal any significant difference among the three Mefloquine groups themselves ( $0.20 < p < 0.25$ ) for falciparum malaria. Analysis of the data based on the positive vivax slide readings showed the five treatment groups to be highly effective in suppressing vivax parasitemias ( $p < 0.0001$ ). Evaluation of results showed Mefloquine to be more efficacious than the standard regimen (S-Py) in suppressing vivax parasitemias ( $p < 0.0001$ ).

One hundred seventy one study subjects who received the S-Py preparation this year and received a sulfone/sulfonamide preparation in one or more past field trials were compared with 23 individuals who received S-Py for the first time this year. Of these twenty-three individuals in the first time S-Py group, one developed a falciparum parasitemia. Sixteen of the 171 individuals experienced a malaria parasitemia: eleven individuals had a vivax

Table 3. P. vivax Parasitemias Experienced by Study Subjects During  
Chemosuppression for 26 Weeks

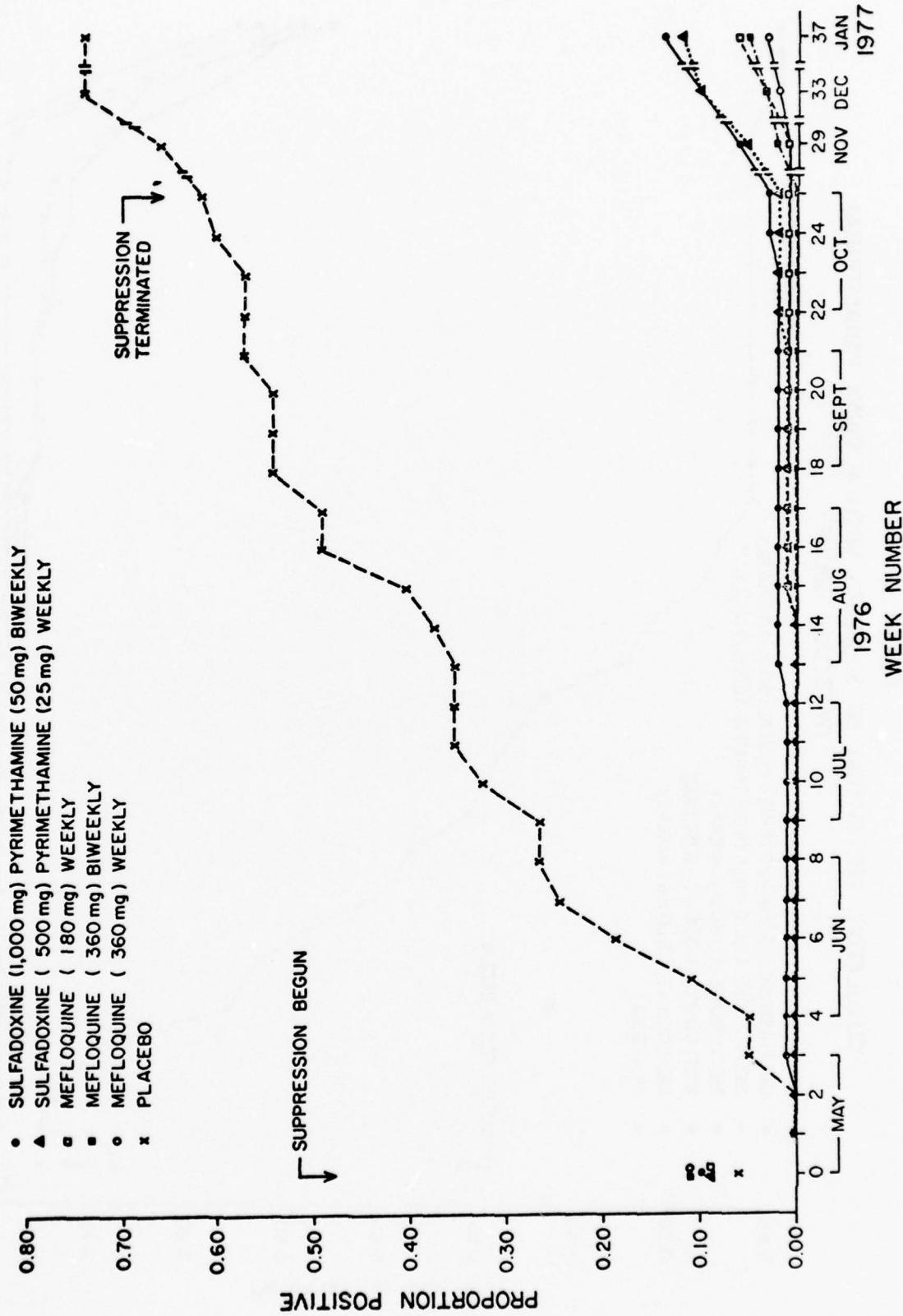
Group	No. Subjects Completing Study	No. (Prop.) Infected	No. Episodes	Average Duration (Weeks) of Episode
S (1000mg)-Py (50mg) fortnightly	162	15 (0.09)	16	2
S (500mg)-Py (25mg) weekly	171	13 (0.07)	14	2
Mefloquine (180mg) weekly	160	3 (0.01)	4	1
Mefloquine (360mg) fortnightly	169	0 (0.00)	0	-
Mefloquine (360mg) weekly	158	1 (0.01)	2	2
Placebo	36	29 (0.80)	51	2

**Table 4. Results of Slide Microscopy\* (Chemosuppressive Phase)**  
**Drug Groups**

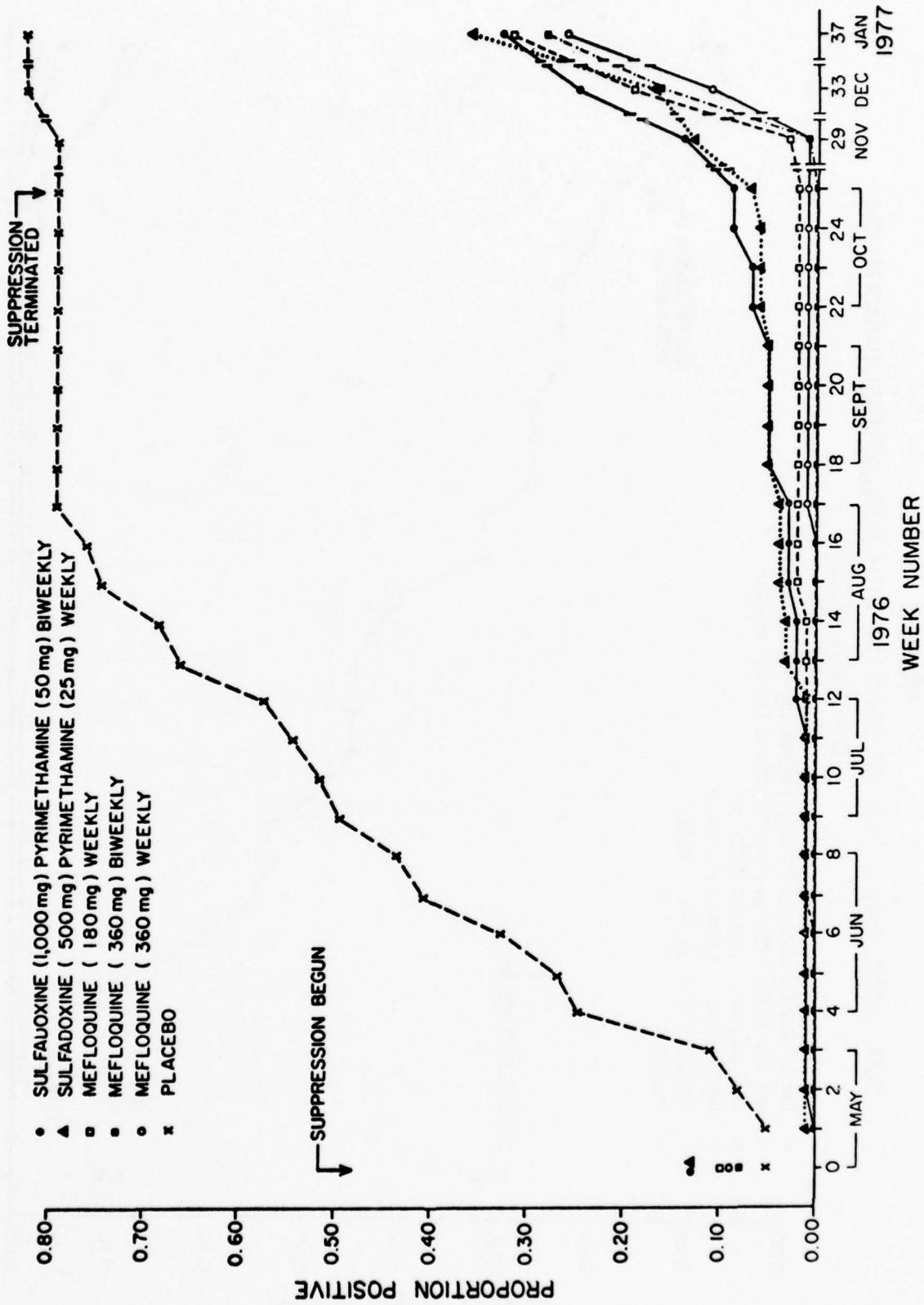
Slide Results	S(1000)-Py(50) fortnightly	S(500)-Py(25) weekly	M(180) weekly	M(360) fortnightly	M(360) weekly	Placebo
Negative	3468	3779	3605	3708	3415	559
Falciparum (P.f.t.) positive	17	10	1	0	2	66
Vivax (P.v.t.) positive	26	29	4	0	4	82
Malariae (P.m.t.) positive	0	0	0	0	0	26
Mixed (P.f.t. + P.v.t.) positive	0	2	0	0	0	5

\*Excluded are P.f.g. results and smears preceeded the period before by absenteeism.

**FIGURE 1**  
**CUMULATIVE PROPORTION OF SUBJECTS WITH *P. FALCIPARUM* PARASITEMIAS**  
**BY STUDY GROUP**



**FIGURE 2**  
**CUMULATIVE PROPORTION OF SUBJECTS WITH *P. vivax* PARASITEMIAS  
 BY STUDY GROUP**



parasitemia, four had a falciparum parasitemia, and one individual had both. Mathematical evaluation using the Fischer Exact Test for a 2 x 2 table showed no significant differences between a group that had repeated exposure to a sulfonamide or sulfone preparation and a group that did not ( $p = 0.2670$ ).

There was no clinical evidence of drug-related adverse effects from the 990 study participants who started the study. There were 30 instances of individuals who had an apparent decrease in hematocrit during the study. These individuals were evenly distributed among the six treatment groups. In most instances, repeat hematocrits a week later were normal and thus suggested a possible laboratory error or very transient condition. Paired information using hematocrits at week 0 and week 24 were analyzed within the treatment groups. Study subjects on the weekly S-Py regimen, the Mefloquine 180 mg weekly, and Mefloquine 360 mg biweekly experienced significant increases in their hematocrits ( $p < 0.01$ ). The results from the other treatment groups were not significant.

There were 631 instances of leucopenia (defined as  $< 4000$  cells/ $\text{ml}$ ). These episodes of leucopenia were distributed among all treatment groups. Statistical evaluation showed a significant difference ( $X^2 = 18.4313$ ;  $p < 0.01$ ) among the groups with the differences appearing in the fortnightly S-Py group and in the placebo group. Further evaluation was undertaken to detect any significant differences in WBC determination following a six month ingestion of medication using paired data from weeks 0 and 26. While most treatment groups experienced a decrease in WBCs, the S-Py fortnightly group experienced the most significant decrease ( $p < 0.003$ ).

There were no significant changes in the measured biochemical parameters.

All study subjects who finished the study were also seen during the follow-up period. Those with parasitemias were treated. Those study subjects with a falciparum parasitemia were given a therapeutic dose of sulfadoxine (1500 mg)-pyrimethamine (75 mg), while those with a vivax or malariae parasitemia were treated with a vivax or malariae parasitemia were treated with the standard regimen using chloroquine (1500 mg over a 3 day period). Primaquine was given for 14 days to those study subjects known to be G-6-PD normal.

2. The Effect of Some Antimalarials on Gametocytogenesis and Sporogony in Human Malaria in Thailand

OBJECTIVE: To determine the effect of several therapeutic regimens on the sexual and sporogonic cycles of natural infections with both P. falciparum and P. vivax.

BACKGROUND: From data collected at this institution and elsewhere, it appears that sulfonamides in general and the sulfadoxine-pyrimethamine combination (Fansidar, Roche) in particular, stimulate gametocyte production in falciparum malaria. Recent it has been suggested that the two and three tablet therapeutic doses have different effects on the sexual forms of the parasite, that two tablets stimulate while three tablets depress falciparum gametocyte production (1). Vivax malaria, which is currently enjoying a dramatic increase in incidence in Thailand, has not been studied in regard to gametocyte response to Fansidar. Since Fansidar has been used in increasing amounts for the therapy of vivax and falciparum infections, it is necessary to know whether vivax gametocyte production responds to Fansidar in a similar fashion to that of falciparum. Although Fansidar is not recommended for vivax infections, it is used widely as self-treatment and as presumptive treatment where species diagnosis cannot be performed.

The new antimalarial, mefloquine hydrochloride, which has been seen to be a highly effective schizonticide administered as a single oral dose, has not been studied in terms of its effect on gametocytes. It is therefore not known whether gametocytocidal therapy must be added to the single-dose schizonticidal regimen.

This study has been designed to test Fansidar and mefloquine in terms of stimulation or suppression of gametocyte production in both falciparum and vivax parasitemias, and to compare these results with those associated with standard therapy - quinine in the case of falciparum and chloroquine in the case of vivax. Additionally, the effect of primaquine as a gametocytocide will be re-evaluated.

Anopheles balabacensis is the primary vector of malaria in Thailand, while Anopheles maculatus is the major vector in Malaysia. This study will also compare the vector efficiency of the two species for vivax and falciparum infections, as well as the effect of these parasites on the longevity of the mosquitoes.

METHODS: The study is currently underway at Phrabuddhabat Hospital, Saraburi Province, Central Thailand. Patients are

admitted to the male medical ward from the hospital out-patient department or from clinics of the National Malaria Eradication Project, both in Phrabuddhabat and in Pak Chong (Nakorn Rajasima Province). Admission criteria for study subjects are:

1. Males at least 18 years of age.
2. Willingness to volunteer for hospitalization and follow-up. The intended procedure is carefully explained to the patient, and he is asked to sign a statement of understanding and agreement.
3. Uncomplicated disease of mild to moderate severity.
4. Asexual parasite count between 1,000 and 100,000/cu.mm.
5. Presence of gametocytes on the initial thick film.

The patients are randomly assigned to one of the following regimens. Each regimen will be administered to twenty patients.

P. falciparum

1. Mefloquine HC1, single dose, 1,500 mg., p.o. (If a patient weighs less than 50 kg., the dose will be 30 mg/kg.)
2. Fansidar, single dose
  - a. 2 tablets (50 mg. pyrimethamine, 1.0 gm. Sulfadoxine)
  - b. 3 tablets (75 mg. pyrimethamine, 1.5 gm. Sulfadoxine)
3. Quinine, iv.v., or p.o., 650 mg. q. 8 hours for seven days.
4. Quinine, 650 mg. q. 8 hours for seven days, plus primaquine 15 mg/day x 5 days.

P. vivax

1. Mefloquine HC1, single dose 1,500 mg., p.o. (or less, as above)
2. Fansidar, single dose
  - a. 2 tablets
  - b. 3 tablets

3. Chloroquine 1,500 mg., total dose p.o.

4. Chloroquine 1,500 mg., total dose p.o. + primaquine  
15 mg/day x 5 days.

The drugs are administered by one of the study physicians.

Upon completion of the 28 day follow-up period, vivax patients are given a course of primaquine 15 mg/day for 14 days. Recrudescences of P. falciparum, if they occur, are retreated with quinine-Fansidar on an individualized basis. Relapses of P. vivax are treated with chloroquine-primaquine.

Sixty laboratory-reared Anopheles balabacensis and 60 Anopheles maculatus (IMR strain) are fed on patients on days 0, 1, 7, 14 and 21. Ten engorged mosquitoes of both species are withheld for determination of longevity. Mosquitoes are also fed on uninfected volunteers for simultaneous control. The mosquitoes are reared and maintained in the AFRIMS Phrabuddhabat insectary. Mosquitoes are dissected 7 and 14 days post-feeding. Guts and glands are examined for oocysts and sporozoites and oocyst indices are determined.

Direct parasite counts, using a modification of the Earle-Perez technique, are performed before admission to the study, twice daily while in the hospital, and at every follow-up visit. Serum sulfonamide, quinine, and mefloquine levels are determined on days of mosquito feeding.

After discharge, patients are taken home in an AFRIMS vehicle and a map to their home is drawn. They are asked to return for follow-up visits on days 7, 14, 21 and 28. If they do not keep their appointments, patients are visited at home and are brought back to Pak Chong Headquarters of NMEP or to the Phrabuddhabat Hospital.

RESULTS: To date 68 patients with P. vivax infection have been admitted to the study and 33 patients with P. falciparum.

#### Therapeutic Results (Table 1)

P. vivax: The mefloquine, chloroquine, and chloroquine-primaquine regimens were alike in their effect on fever clearance and asexual parasite clearance. In the two groups treated with Fansidar, unacceptable therapeutic results have been obtained. The two tablet-dose of Fansidar eliminated initial asexual parasitemia in only six of ten patients studied. Fever clearance

TABLE 1  
Results of Therapy

P. vivax

Treatment	Number of Patients	Mean Initial Asexual Count	Mean Fever Clearance Time (hours)	Mean Asexual Parasite Clearance Time (hours)
Mefloquine	15	5,324	36	46
Fansidar (2)	10	8,435	69	73*
Fansidar (3)	8	7,283	47	90
Chloroquine	18	5,832	40	52
Chloroquine- Primaquine	17	7,064	48	42

P. falciparum

Treatment	Number of Patients	Mean Initial Asexual Count	Mean Fever Clearance Time (hours)	Mean Asexual Parasite Clearance Time (hours)
Mefloquine	5	23,732	48	84
Fansidar (2)	7	11,332	87	73
Fansidar (3)	4	13,050	68	72
Quinine	9	15,351	51	68
Quinine- Primaquine	8	14,192	57	80

\*

Four patients in this group had not cleared asexual parasitemia by Day 7. This value is the mean of those which did clear before Day 7.

and parasite clearance in the remainder of the patients was very sluggish (Table 2). Three tablets of Fansidar was successful in clearing parasitemia in all of eight patients studied, however the parasite clearance time was prolonged at 90 hours.

P. falciparum: Since only 33 patients infected with P. falciparum have been studied to date, there are too few patients in each group to compare therapeutic efficacy.

#### Gametocyte Counts (Table 3)

P. vivax: Initial gametocyte counts in the five groups were comparable and their rates of disappearance paralleled those of the asexual parasites. It is interesting to note, however, that gametocytes persisted in the two-tablet Fansidar group for a longer period on the average than in any other group. This reflects the fact that Fansidar was not successful in eliminating initial parasitemia in four of the ten patients studied. In addition, the mean gametocyte count of the eight patients treated with three tablets of Fansidar seemed to be higher than in any other group. Whether this represents stimulation by the drug is not yet clear.

P. falciparum: It is noteworthy that once again falciparum patients treated with Fansidar showed much higher peak gametocytemias than patients in the other treatment groups. It is too early to comment on other features of this portion of the study.

#### Mosquito Feeding

P. vivax: Fifty-four percent of patients studied to date have been infectious to vector mosquitoes before treatment (Table 4). It appears that overall Anopheles balabacensis is more likely to be infected with P. vivax than Anopheles maculatus. Mefloquine and Fansidar-treated patients are apparently equally infectious to mosquitoes on Day 1 following therapy. The chloroquine and the chloroquine-primaquine treated groups were much less likely to infect mosquitoes of either species on the day following therapy.

P. falciparum: It is too early to evaluate mosquito feeding data from this group of patients, however, it may be noted that two tablets of Fansidar gave rise to a number of positive mosquito feeds on Day 7, 14 and 21 following therapy.

TABLE 2

Results of *P. vivax* Therapy Using  
Two Tablets of Fansidar

Patient No.	Initial Asexual Count	Mean Fever Clearance time (hours)	Mean Asexual Parasite Clearance time (hours)	Comment
11-0348	34,390	42	53	
11-0353	6,300	80	No clearance by Day 7*	
11-0355	4,452	30	64	
11-0366	13,800	80	No clearance by Day 7*	
11-0367	8,310	No fever	52	
11-0376	3,080	72	No clearance by Day 7*	
11-0388	5,796	120	102	Relapse Day 21*
11-0399	1,160	28	20	
11-0400	1,700	96	140	Relapse Day 28*
11-0401	5,360	72	No clearance by Day 7*	

\* Patients were retreated with chloroquine-primaquine

TABLE 3  
Mean Gametocyte Counts on Days of Mosquito Feeding

Drug	Day				
	0	1	7	14	21
<u>P. vivax</u>					
Mefloquine	186	93	0	0	0
Fansidar (2)	368	90	100	5	0
Fansidar (3)	431	250	0	0	0
Chloroquine	230	55	0	0	0
Chloroquine- Primaquine	128	26	0	0	0
<u>P. falciparum</u>					
Mefloquine	163	160	220	30	15
Fansidar (2)	3	60	350	794	88
Fansidar (3)	3	10	1,580	1,490	620
Quinine	29	50	60	10	-*
Quinine- Primaquine	68	260	-*	-*	-*

\*

No feeds - no gametocytes or lost to follow-up.

TABLE 4  
Summary of Mosquito Feeding Results

P. vivax

Therapy	Day of Feed	A. balabacensis Positive Feed	A. maculatus Positive Feed
Mefloquine	0	7/17	41%
	1	4/14	29%
	7	0/0	-
	14	0/0	-
	21	0/0	-
Fansidar (2)	0	2/10	20%
	1	4/11	36%
	7	1/3	33%
	14	0/1	0%
	21	0/0	-
Fansidar (3)	0	6/8	75%
	1	3/6	50%
	7	0/0	-
	14	0/0	-
	21	0/0	-
Chloroquine	0	7/17	41%
	1	2/11	18%
	7	0/0	-
	14	0/0	-
	21	0/0	-
Chloroquine- Primaquine	0	2/11	64%
	1	0/10	0%
	7	0/0	-
	14	0/0	-
	21	0/0	-

TABLE 5  
Vector Engorgement and Survival

P. vivax

Therapy	Day	No. of Lots Fed	Anopheles % Engorgement	Anopheles % Survival	Anopheles % Engorgement	Anopheles % Survival
<b>Mefloquine</b>	0	17	70	66	62	48
	1	15	75	75	74	53
	7	6	77	55	53	57
	14	3	68	65	59	62
	21	4	74	63	54	57
<b>Fansidar (2)</b>	0	10	78	73	72	67
	1	11	83	66	86	58
	7	3	84	71	72	64
	14	2	60	66	53	32
	21	0	-	-	-	-
<b>Fansidar (3)</b>	0	8	80	89	69	55
	1	6	67	61	83	51
	7	3	91	49	63	50
	14	2	71	71	53	43
	21	1	80	74	78	100
<b>Chloroquine</b>	0	17	82	85	71	53
	1	13	82	69	81	61
	7	3	60	67	63	55
	14	2	78	78	75	54
	21	2	83	69	75	50
<b>Chloroquine- Primaquine</b>	0	11	77	83	77	56
	1	10	85	72	84	53
	7	3	86	90	66	45
	14	2	44	90	58	48
	21	0	-	-	-	-

TABLE 6  
Longevity in P. vivax-infected and Uninfected Vector Mosquitoes

Mosquitoes fed before patient treatment	No. of lots	<u>Anopheles balabacensis</u>		<u>Anopheles maculatus</u>	
		Infected	Uninfected	Infected	Uninfected
	No. of lots	22	19	20	13
	Longevity (day)	Mean	21.3	23.5	16.9
		Range	1-48	1-51	2-34
Mosquitoes fed one day after patient treatment	No. of lots	9	8	11	10
	Longevity (day)	Mean	26.4	29	19.6
		Range	5-48	6-53	4-40

### Vector Engorgement and Survival

P. vivax: The percentage of mosquitoes taking a bloodmeal upon being exposed to infected patients was similar in all treatment categories (Table 5). In nearly every case larger numbers of Anopheles balabacensis fed than Anopheles maculatus. Likewise, larger numbers of Anopheles balabacensis survived to be dissected than Anopheles maculatus. These data suggest that Anopheles balabacensis may be a more effective vector of vivax malaria from Thailand.

Mosquitoes were held separately for longevity analysis in order to compare the effect of the parasite on the vector. Mosquitoes fed on uninfected volunteers throughout the course of the study survived a mean length of 26.4 days (A. balabacensis) and 18.4 (A. maculatus).

Mosquitoes from infected lots were compared with those fed at the same time on normal volunteers (simultaneous controls). A. balabacensis from infected and control lots showed little difference, but uninfected A. maculatus appear to have significantly greater longevity than those from infected lots.

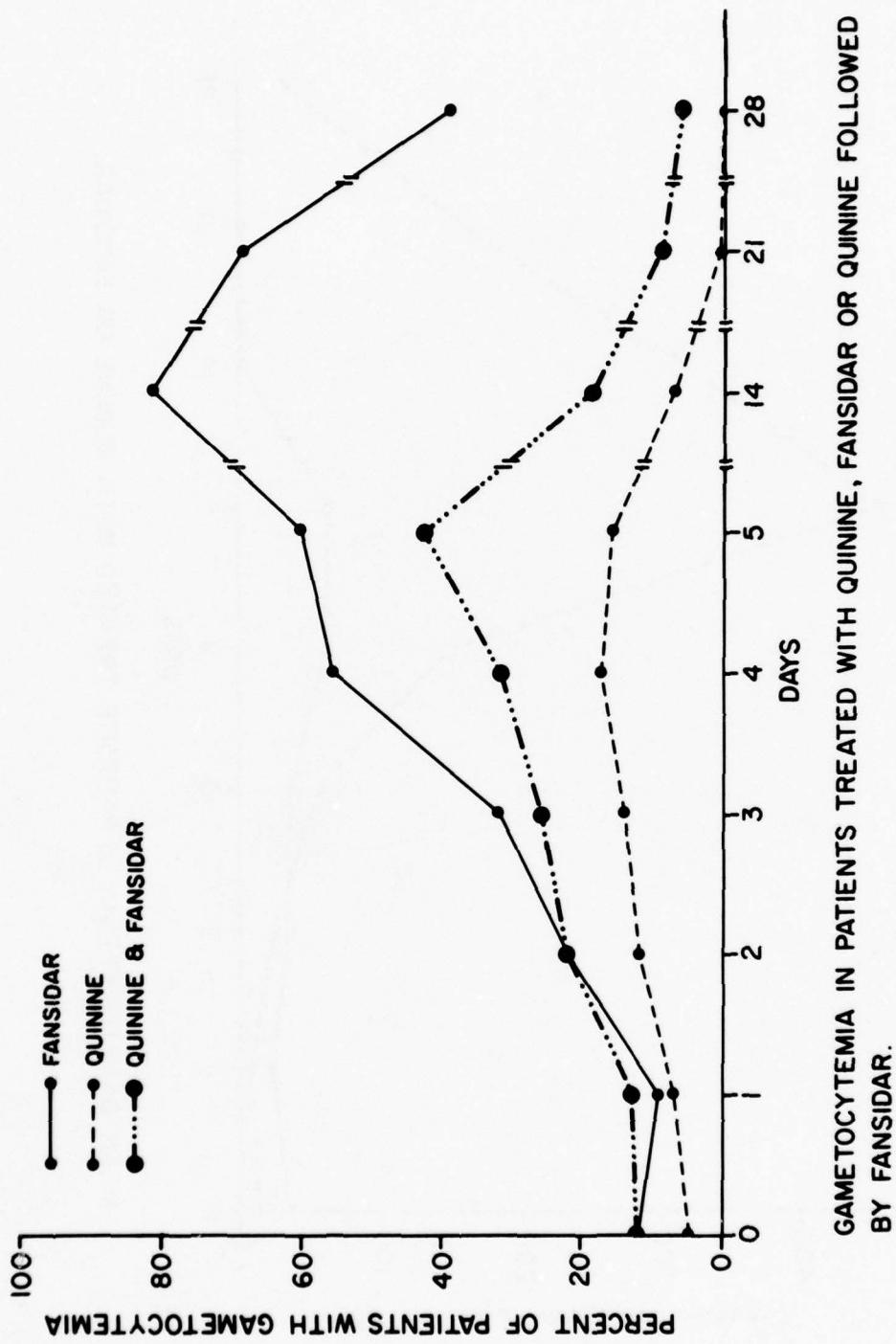
P. falciparum: It is too early to completely evaluate data from this group of patients. However, it seems that Anopheles balabacensis may again be a more efficient vector of P. falciparum, as seems to be the case with P. vivax.

Data is still being collected in this study, and it is anticipated that at least another six months will be required for completion.

### 3. P. falciparum Gametocytemia Following Schizonticidal Treatment

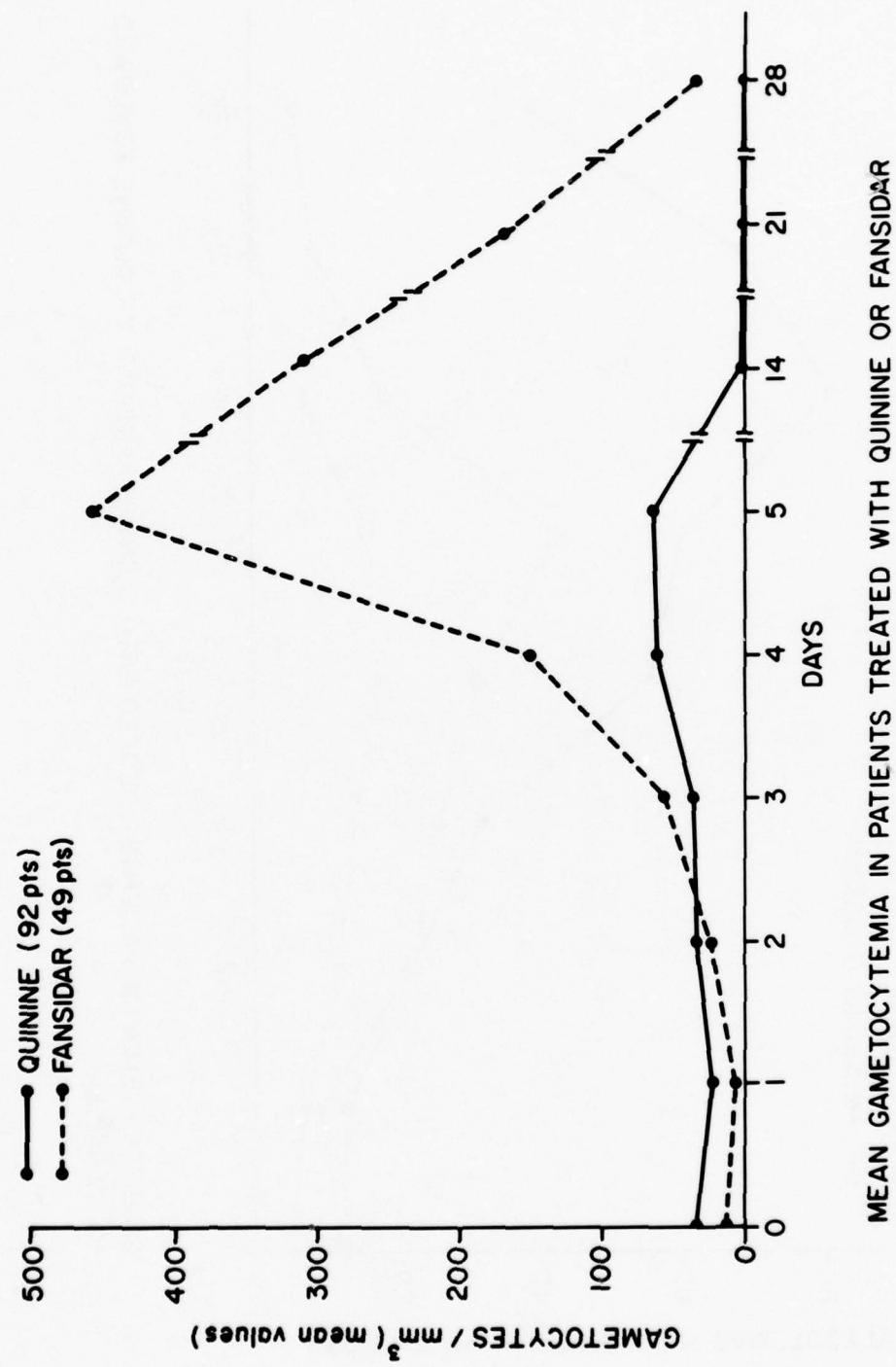
OBJECTIVE: To document the effect of various antimalarials upon the production of gametocytes.

BACKGROUND: Sulfonamide antimalarials, for example, sulfalene and sulfadoxine, have been associated with an apparent stimulation of P. falciparum gametocytemia. Administration of sulfalene at the time of gametocyte genesis (approximately 10 days before the appearance in the peripheral blood) has been shown to stimulate gametocyte production but is thought to render them sterile. Sulfalene given closer to the time of gametocyte patency does not sterilize the gametocytes and they remain infective to vector mosquitoes (2).



GAMECTOCYTEMIA IN PATIENTS TREATED WITH QUININE, FANSIDAR OR QUININE FOLLOWED BY FANSIDAR.

FIGURE 1



MEAN GAMETOCTYEMIA IN PATIENTS TREATED WITH QUININE OR FANSIDAR

FIG. 1

In view of the widespread use of Fansidar (sulfadoxine-pyrimethamine) for malaria in Thailand and in other areas of the world where the parasite is resistant to chloroquine, it is important to know the effect upon gametocytemia of this drug combination. If the drug is shown to be stimulatory, then the use of a gametocytocide such as primaquine is mandatory in any rational control scheme. Quinine is considered to have no effect upon gametocytemia in P. falciparum.

METHODS: Retrospective analysis of data obtained from patients treated at the Trad Provincial Hospital in 1973 and 1974 was accomplished. The original study was an evaluation of the therapeutic efficacy of three antimalarial regimens, Fansidar alone, quinine alone, and a course of quinine followed by a dose of Fansidar. The three groups were analyzed in terms of the percentage of patients developing blood films positive for gametocytes and gametocyte levels reached following therapy.

RESULTS:

a. Numbers of patients developing gametocytes (Figure 1). On admission to the study patients from the three groups were alike in the prevalence of gametocyte-positive smears. However, the Fansidar group subsequently showed a dramatic increase in the incidence of gametocyte positive smears. On Day 14, 82% of patients were positive in the Fansidar group whereas only 7% were positive for gametocytes in the quinine group.

b. Gametocyte counts in patients following antimalarial therapy (Figure 2). Peak gametocytemia was seen in all groups on Day 5 following therapy. However, there was striking difference in gametocyte levels attained. The Fansidar group had a mean value of 460 gametocytes per cu.mm., whereas the quinine group had a mean value of only 65/cu.mm.

Retrospective analysis of data obtained from patients treated with other antimalarials is being examined for comparison with those reported here.

4. Single-Dose Therapy of Falciparum Malaria With Mefloquine or Fansidar(R) (Pyrimethamine-Sulfadoxine)

OBJECTIVE: To further evaluate the therapeutic efficacy of mefloquine hydrochloride given as a single dose to patients

with acute falciparum malaria. To compare its effect and toxicity with that of Fansidar, the current single-dose drug of choice.

BACKGROUND: Mefloquine hydrochloride (WR142490) is an analog of quinine and of the investigational drug WR30090 which was used successfully to treat cases of chloroquine-resistant falciparum malaria in American servicemen returning from the Vietnam war as well as indigenous cases in Thailand. Mefloquine has been studied in Thailand both as a single regimen and in combination with quinine. It also has been studied as a suppressant of falciparum and vivax infections in the Bu Phram Valley, Prachinburi Province. It has been shown to be highly effective both as therapy and prophylaxis.

METHOD: The study was carried out at Phrabuddhabat Hospital in Saraburi Province, Central Thailand. This area is known to be endemic for chloroquine-resistant falciparum malaria. Current in vitro data confirm the high level of chloroquine resistance of parasites in the patients reporting for treatment in this area. Patients were selected from those presenting to the hospital out-patient department and the Passive Detection Center of the National Malaria Eradication Project Regional Headquarters in Phrabuddhabat.

Criteria for selection of patients included the following:

1. Males at least 18 years of age (possible side effects of neither drug have been completely evaluated in pregnancy or in young children).
2. Moderate parasitemias. Patients were generally not admitted to the study if the asexual count was less than 1,000 or more than 100,000/cu.mm.
3. Uncomplicated disease. Patients in coma, with severe jaundice, uncontrollable vomiting or with evidence of renal, pulmonary, or cardiac complications were not admitted to the study.
4. Willingness to sign a consent form after details of the study had been explained. The use of a new drug, the need for prolonged follow-up and repeated venipuncture were described to the patients before the signature was requested. A Thai language information sheet containing details of the study was given to the patients. Patients considered eligible for the study were admitted to the male medical ward of the hospital and were followed

by one of the investigators with clinical rounds conducted at least twice daily. Quantitative parasite counts by the technique of Earle & Perez were performed eight hourly until asexual parasitemia had disappeared, and at each follow-up visit. A detailed record of symptoms and physical signs was kept by the physician during hospitalization and at follow-up visits. In addition, clinical laboratory examination of hematologic, renal and hepatic function were performed on admission and at intervals during hospitalization and follow-up. Serum screening for quinine and sulfonamide was performed at AFRIMS, Bangkok, on specimens obtained on admission and at follow-up visits in order to detect additional antimalarial self-treatment.

Patients were kept in the hospital an average of four days and then followed as out-patients on days 10, 15, 21, 28, 38, 50 and 60 after admission. Upon discharge, patients were driven home and a record was kept of their home address. If a patient failed to keep a follow-up appointment, an attempt was made to follow him at home. Mefloquine was supplied by the Walter Reed Army Institute of Research and the Fansidar\* was purchased locally. Drugs were administered by an investigator and patients were checked to assure that they had swallowed the tablets. Alternate patients were assigned to one of the following single-dose regimens:

1. Fansidar three tablets (pyrimethamine 75 mg., Sulfadoxine 1.5 gm.)

2. Mefloquine hydrochloride six tablets (1.5 gm)

In addition, antipyretics and analgesics, intravenous fluids and suppressants of nausea and diarrhea were used as clinically indicated.

Treatment results were evaluated according to the WHO criteria originally conceived to evaluate chloroquine resistance: "S" indicates clearance of asexual parasitemia and maintenance of a negative blood film for 28 days after therapy. "RI" refers to initial clearance of parasitemia followed by recrudescence within 28 days after treatment, "RII" indicates initial reduction in the level of parasitemia, but failure to clear in seven days. "RIII" indicates no reduction in parasitemia following treatment.

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\* Hoffmann La Roche Company

**RESULTS:** Ninety-one patients were admitted to the study and followed for at least seven days. Of these, 75 were followed for at least 28 days (82%) and 50 (55%) were followed for 60 days.

There was no significant difference between the two groups in regard to age, initial parasite count or percentage returning to areas of transmission during the period of follow-up.

In patients followed at least 28 days, 100% of the mefloquine group (37/37) were radically cured ("S" response). Of the Fansidar group, 89% were cured (34/38); there were two RI failures and two RII failures (Table 1). RI recrudescences were detected on Day 21 and Day 28 of follow-up respectively, and may have been due to reinfection, although the patients denied having returned to areas of malaria transmission. After recrudescence, treatment with mefloquine resulted in radical cure in both cases. Patients described as experiencing RII failures were retreated with mefloquine after Fansidar had failed to clear parasitemia in seven days. Both of these patients cleared their parasitemia rapidly and remained well for an additional 60 days of follow-up.

Fever and parasite clearance times for mefloquine (58 and 62 hours, respectively) were shorter than those for Fansidar (64 and 67 hours) but the difference was not significant. The fever and parasite clearance times associated with mefloquine were slightly better than those obtained when quinine is used alone, (64 and 70 hours respectively). Fansidar rates were almost identical with those for quinine.

The regimens were alike in their association with symptoms of malaria; e.g., headache and dizziness, but differed in regard to the presence of gastrointestinal complaints (Table 2). Nausea, vomiting and diarrhea were more common in the mefloquine group than in the Fansidar group. However, no patient had severe vomiting or diarrhea, and these symptoms were easily managed with routine medication.

There was no significant difference between the two groups either before or during therapy, in routine hematologic or biochemical parameters.

Abnormalities which did occur were more likely related to malaria than to either therapeutic regimen, and most had cleared by the end of the follow-up period.

RESULTS OF TREATMENT

Table 1

Regimen	Number of Patients	Mean Asexual Parasite Count	Mean Fever Clearance Time (hours)	Mean Parasite Clearance Time (hours)	Therapeutic Result			Cure Rate
					RI	RII	RIII	
Fansidar	38	25,770	64	67	2	2	0	34
Mefloquine	37	24,750	58	62	0	0	0	37 100%

Table 2  
SYMPTOMS ENCOUNTERED DURING INITIAL FOUR DAYS  
OF HOSPITALIZATION

Symptom	Regimen			
	Fanidar (48 pts.)		Mefloquine (49 pts.)	
	Number	Percent	Number	Percent
Headache	45	94%	46	94%
Dizziness	44	92%	47	96%
Nausea	28	58%	41	84%
Vomiting	14	29%	25	51%
Diarrhea	8	17%	13	27%

## 5. Mefloquine Pharmacokinetics Following Oral Administration

OBJECTIVE: To determine the pattern of absorption and elimination of orally administered doses of mefloquine.

BACKGROUND: A technique for accurate chemical analysis for mefloquine in blood specimens is now available in the laboratories of the Department of Pharmacology at the Walter Reed Army Institute of Research. This provides an opportunity to develop detailed pharmacokinetic information from use of the drug in humans. Data has been collected in the United States from normal, uninfected volunteer subjects, who were given several different oral dosages of mefloquine. However, no data on the absorption and the elimination patterns of this antimalarial in human subjects infected with P. falciparum malaria have hitherto been collected. Information currently available indicates that the drug does have a prolonged biologic half-life in man (15-25 days) which probably accounts for its efficacy as a single-dose therapeutic, and also its success as a chemo-suppressant.

Data collected both in the United States and in Thailand have already shown that mefloquine is a safe and effective anti-malarial.

METHODS: Fifteen subjects were admitted to the study, selected from those presenting to the hospital out-patient department in Phrabuddhabat, Saraburi Province, Central Thailand, as well as the Passive Detection Center of the National Malaria Eradication Project in Phrabuddhabat. The patients were presumably infected with a chloroquine-resistant strain of P. falciparum, since current in vitro data suggests that chloroquine-sensitive strains are no longer seen in the Phrabuddhabat area. Patient selection criteria were identical to those used in previous mefloquine studies; i.e.,

1. Males at least 18 years of age.
2. Moderate parasitemia (greater than 1,000 less than 100,000/cu.mm.).
3. Uncomplicated disease.
4. Willingness to sign a consent form for the use of a new drug.

Patients considered eligible were admitted to the medical ward of Phrabuddhabat Hospital and were followed by one of the investigators with clinical rounds conducted at least twice daily. Parasite counts were performed three times daily and a detailed record was kept of signs and symptoms. Patients were kept in the hospital an average of four days, and then followed as outpatients for up to 84 days following therapy. A total of 32 blood specimens per subject was obtained over this period of twelve weeks. Sampling times were selected to minimize the variance estimate of the slope of each of the three kinetic phases anticipated. The long duration of this study was required to provide specimens for analysis at three to four times the elimination half-life of the drug.

RESULTS: Fifteen patients were studied according to the protocol and all were clinically and parasitologically cured. Specimens of heparinized whole venous blood were collected and have been sent to the Division of Medicinal Chemistry, Walter Reed Army Institute of Research in Washington. Analysis of the samples is currently underway and results are pending.

6. In vitro Chloroquine and Quinine Sensitivity of a Thai Strain of Plasmodium falciparum

OBJECTIVE: To document chloroquine susceptibility of P. falciparum using the Rieckmann in vitro technique in Phrabuddhabat, the site of current therapeutic drug trials.

BACKGROUND: The problem of chloroquine-resistant P. falciparum was first described in Thailand in 1962. It is currently believed that sensitive strains have all but disappeared from Thailand. The determination of drug resistance in P. falciparum based on the clinical response in antimalarial agent has several limitations. Rieckmann, et al., in 1968 described a relatively simple and rapid technique for the in vitro detection of chloroquine resistant falciparum malaria. In Thailand, Colwell in 1972 used this technique in describing chloroquine-resistant strains of P. falciparum in several parts of Thailand.

METHODS: Subjects for this study were selected from patients presenting to the hospital out-patient department or to the Malaria Eradication Project Clinic in Phrabuddhabat District, Saraburi Province, Central Thailand. Twelve to fifteen milliliters of blood was obtained from suitable patients by venipuncture and ejected into a sterile Erlenmeyer flask containing a number of glass beads. This specimen was swirled for 15 minutes in

Figure 1. Effects of Quinine *in vitro* upon Uganda I, Malayan (Camp), and Thai strains of *P. falciparum*

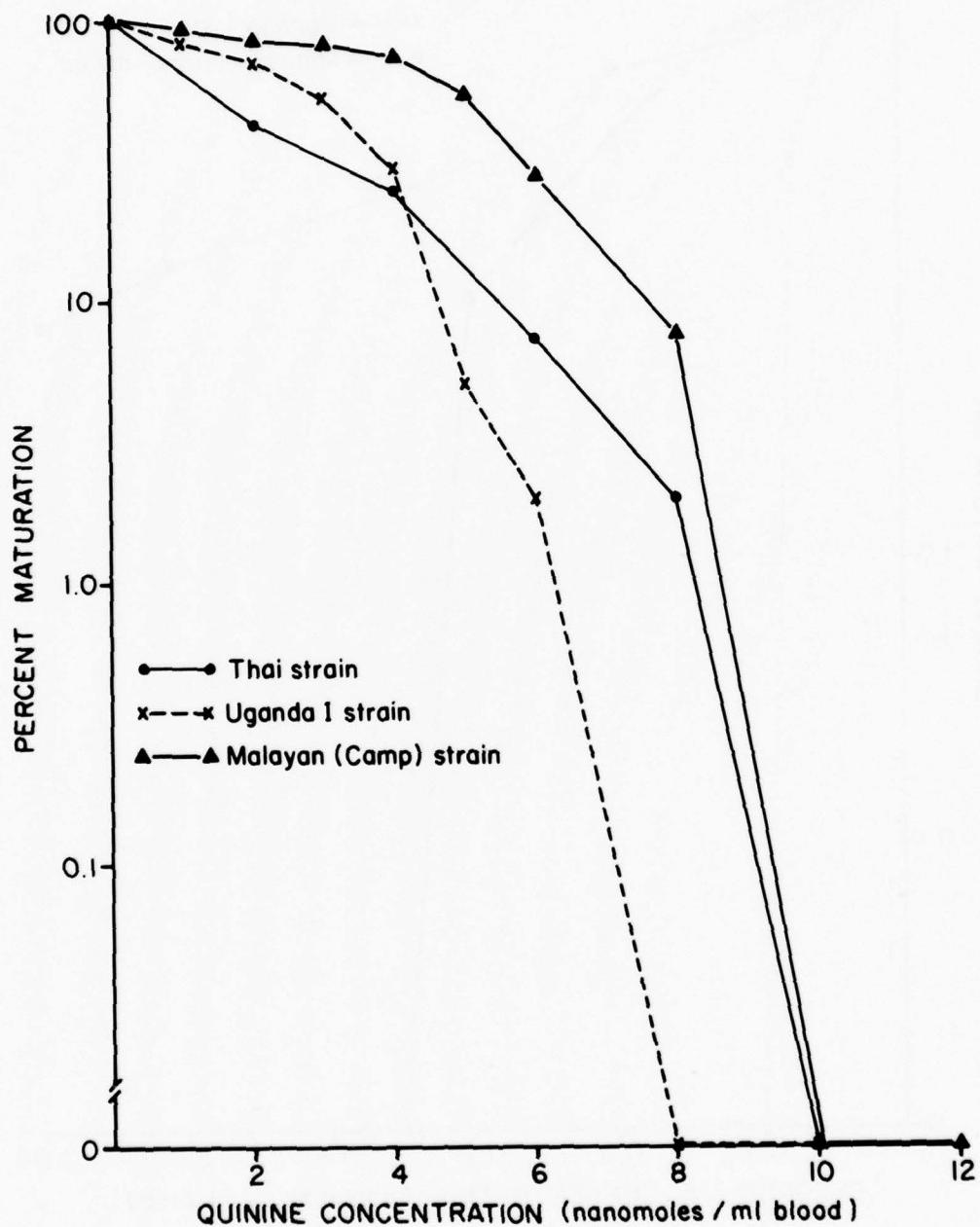


FIGURE 1

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Figure 2 Effects of Chloroquine in vitro upon Uganda I, Malayan (Camp), and Thai strains of P. falciparum

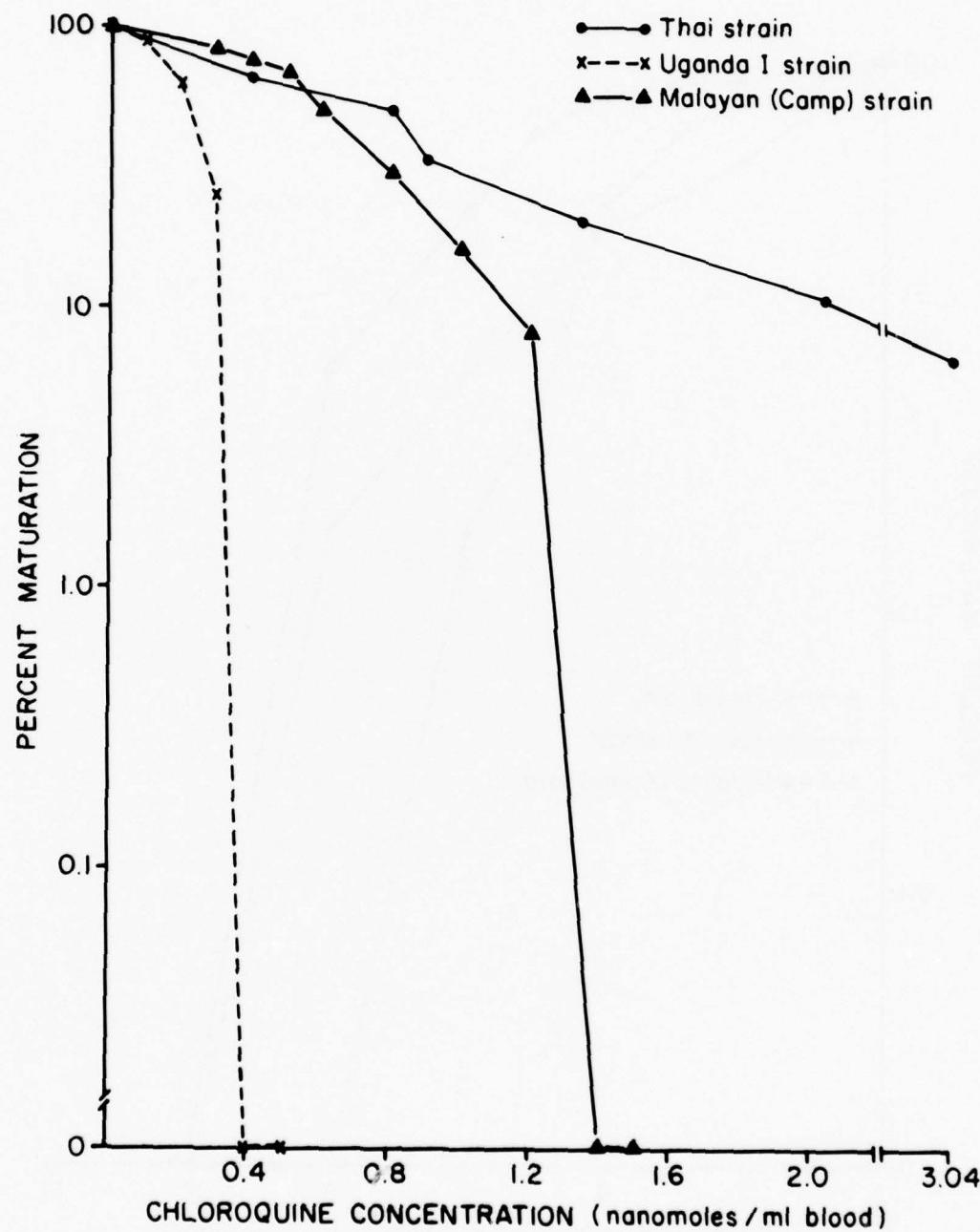


FIGURE 2

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order to defibrinate the blood. One milliliter of the blood was pipetted into each of several screw capped flat bottom glass vials containing glucose and varying concentrations of chloroquine or quinine. The contents were mixed and incubated in a water bath for 24 hours. At the end of incubation thick blood films were made, coded and dried overnight. The films were stained with Giemsa for 30 minutes. Examination of the smears was accomplished without the reader's knowledge of the relative position on the slide of smears from control or drug-treated vials. The degree of maturation was assessed by counting the number of schizonts with two or more nuclei per 200 consecutive asexual parasites. Values obtained for the vials were divided by the values obtained for corresponding control vials, and the results expressed as percentage maturation.

RESULTS: Twenty-nine samples in each group were successfully cultured.

a. Quinine. Samples of blood containing two nanomoles of quinine showed little or no inhibition of the formation of schizonts but when the samples contained four nanomoles or more slight to complete inhibition of maturation was observed. Complete arrest of the formation of schizonts was observed in all samples containing ten nanomoles of quinine per milliliter of blood. Comparison of quinine sensitivity of the Phrabuddhabat strain with the reference sensitive Uganda I and resistant Malayan (Camp) strains of *P. falciparum* shows nearly identical susceptibility to quinine (Figure 1).

b. Chloroquine. Samples of blood containing 0.4 to 1.35 nanomoles of chloroquine showed little or no inhibition of maturation. When the samples contained 2.03 and 3.04 nanomoles marked inhibition of maturation was observed but the formation of schizonts was not completely inhibited. Only 44.8 and 72.4 percent of samples containing 2.03 and 3.04 nanomoles of chloroquine respectively were completely inhibited. In comparison with the sensitive Uganda I strain and the moderately resistant Malayan (Camp) strain, the Thai parasite was highly resistant (Figure 2).

This study is complete, however it is planned that *in vitro* susceptibility of *P. falciparum* to amodiaquine and mefloquine will be evaluated in the future, and that the effect of chloroquine will be monitored continually in areas where drug trials are underway.

7. Evaluation of Experimental Antimalarial Drugs in Rhesus Monkeys Infected with Plasmodium cynomolgi (Blood Schizonticidal Tests)

OBJECTIVE: To evaluate blood schizonticidal activity of selected experimental drugs against P. cynomolgi malaria in rhesus monkeys. These studies are coordinated by the Division of Medicinal Chemistry, Walter Reed Army Institute of Research, and the results are used to aid in the selection of more effective antimalarial drugs for human use.

BACKGROUND: This is a continuation of studies initiated in 1971. A chronological report of methodology and results are available in SEATO Medical Research Laboratory Annual Reports for 1971 through 1976.

METHODS: Experimental drugs were evaluated in rhesus monkeys (Macaca mulatta) utilizing various dosage levels (mg/kg). Only those monkeys found to be free of malarial parasites on prestudy examination were utilized, a total of 187 rhesus. Each animal was infected by intravenous inoculation of  $5 \times 10^8$  parasitized erythrocytes obtained from donor monkeys infected with P. cynomolgi strain B. Post-inoculation day 4, test drugs were administered orally (by gastric intubation) for seven days. The monkeys were bled to determine their level of parasitemia daily for the first fifteen days following inoculation and every other day for the remainder of the study. Suppression of parasitemia was indicative of blood schizonticidal activity. Post-treatment day 20, splenectomy was performed on monkeys in which there was no evidence of parasitemia. Monkeys positive for malaria parasites were terminated. Splenectomized monkeys continuously negative for malaria parasites through post-treatment day 50 were considered cured.

Table 1. Summary of Blood Schizonticidal Tests in Rhésus Monkeys

Type of Compound	WRAIR Drug Number	Minimum Curative Dose (mg/kg/day)
4 - Aminoquinoline	219774 225449	1.0 1.0
8 - Aminoquinoline	225448 228710 231030 231033 231530	1.0 1.0 1.0 1.0 1.0
2, 4 - Diaminoquinazoline	148799 199361 206891 222448 223143 225329 226337	3.16 3.16 *NC (31.6) 0.1 0.316 1.0 1.0
Quinoline methanols	226253 226663 228974 229561	10.0 3.16 31.6 31.6
Phenanthrenemethanol	181613	*NC (31.6)
Miscellaneous	49808	*NC (31.6) (Administered I.M.)

\* Not Curative. The compound had suppressive activity but did not cure at the maximum dose tested. The maximum tested dose is indicated in parentheses.

## 8. Evaluation of Experimental Antimalarial Drugs for Radical Curative Activity in Rhesus Monkeys

OBJECTIVE: To evaluate the radical curative effectiveness of selected experimental antimalarial drugs in rhesus monkeys (Macaca mulatta) infected with Plasmodium cynomolgi malaria.

BACKGROUND: Studies to evaluate antimalarial drugs in rhesus monkeys began in 1971. Initial projects were designed to test suppressive (blood schizonticidal) activity. In 1974 a project was initiated with the goal of establishing an intramural capability to evaluate drugs for radical curative activity (sporozoite induced testing). These are continuing studies which are conducted in association with the Division of Medicinal Chemistry, Walter Reed Army Institute of Research.

METHODS: Test rhesus were infected with sporozoites produced in Anopheles balabacensis mosquitoes. Mosquitoes four to five days old, divided into lots of 400, were starved approximately four hours, then offered a blood meal on an intact rhesus acutely infected with P. cynomolgi. The feeding was scheduled to coincide with (1) the second or third rise in parasitemia. and (2) the presence of both male and female gametocytes. Mosquitoes were examined on post-feed day 6 for gut oocysts; 20 to 80 per gut were considered optimal for sporozoite development. On post-feed day 13 an estimate was made of the sporozoite concentration per salivary gland pair. The following day sporozoites were harvested and diluted in saline; normal monkey serum (1:1) to a concentration of  $5-20 \times 10^5$  per ml. Each test rhesus was inoculated intravenously with 1 ml.

Parasitemia developed in eight days. After parasitemia reached  $5-25 \times 10^5$  per cmm, approximately 11 to 15 days, drugs were administered daily for seven days on the basis of mg/kg body weight. To permit evaluation of drug activity against tissue parasitic forms independently of blood schizonticidal activity, chloroquine phosphate was administered simultaneously with each test drug at 5.0 mg/kg/day.

Following administration of the test drug, blood was monitored by examination of giemsa stained blood smears daily for twelve days and every two days thereafter. Monkeys which remained negative on post-treatment day 20 were splenectomized and monitored an additional 33 days. Splenectomized monkeys which remained free of malaria parasites through post-treatment day 53 were considered cured.

Summary of Sporozoite Induced Tests

Type of Compound	WRAIR Drug Number	Minimum Curative Dose ** (mg/kg/day)
8-aminoquinoline	6020	10.0
	6026	1.0
	181023	1.0
	184118	*NC (3.16) (toxic at 10.0)
	211666	3.16
	212293	NC (3.16)
	216100	3.16
	216643	NC (10.0)
	216644	NC (10.0)
	218573	NC (10.0)
	219423	NC (1.0) (toxic at 10.0)
	223442	1.0
	223658	1.0
	223747	10.0
	223756	1.0
	224097	0.316
	224382	3.16
	224398	NC (3.16) (toxic at 10.0)
	224486	1.0
	225503	NC (3.16)
	225635	NC (10.0)
	225742	NC (10.0)
	226256	1.0
	226257	NC (1.0) (toxic at 10.0)
	226292	1.0
	226296	NC (1.0) (toxic at 3.16)
	226393	10.0
	226394	10.0
	226426	0.316
	226573	1.0
	226619	3.16
	226762	10.0
	226937	3.16
	227175	NC (10.0)
	227495	1.0
	228327	3.16
	228456	0.316
	228457	1.0
	228708	1.0
	228710	1.0
	229431	NC (10.0)
	230388	NC (1.0) (toxic at 10.0)
	230837	NC (10.0)

\* Not Curative. The compound did not cure at the maximum dose tested or tolerated. The maximum dose is indicated in parentheses.

\*\* Administered orally with 5.0 mg/kg/day of chloroquine phosphate.

Summary of Sporozoite Induced Tests (continued)

Type of Compound	WRAIR Drug Number	Minimum Curative Dose ** (mg/kg/day)
8-aminoquinoline	231163 231350 231530 231776 232147	10.0 NC (10.0) 0.316 10.0 NC (10.0)
Naphthyridinones	206287 222119 231138 231165 232144 232179	NC (10.0) NC (10.0) 10.0 NC (10.0) NC (10.0) NC (10.0)
Pteridines	199361 206891	NC (10.0) NC (10.0)
Quinazoline	222448 225329	NC (10.0) NC (10.0)
Quinolines	229092 229011 228258	NC (10.0) NC (10.0) NC (10.0)
Hypoxanthines	226901 226902 229046	NC (10.0) 10.0 NC (10.0)
Miscellaneous	5601 119621 129455 148799 158124 182058 204585 206513 216693 220594 220679 222683 225449 226626	NC (10.0) NC (10.0)

\* Not Curative. The compound did not cure at the maximum dose tested or tolerated. The maximum dose is indicated in parentheses.

\*\* Administered orally with 5.0 mg/kg/day of chloroquine phosphate.

Summary of Sporozoite Induced Tests (continued)

Type of Compound	WRAIR Drug Number	Minimum Curative Dose ** (mg/kg/day)
Miscellaneous	228769	NC (10.0)
	230216	NC (10.0)
	230225	NC (10.0)
	230284	NC (10.0)
	230386	NC (10.0)
	230621	NC (10.0)

\* Not Curative. The compound did not cure at the maximum dose tested or tolerated. The maximum dose is indicated in parentheses.

\*\* Administered orally with 5.0 mg/kg/day of chloroquine phosphate.

RESULTS: Problems encountered in sporozoite production were rectified and procedures for production and quantitation of sporozoites were standardized. Therefore, the goal of establishing a radical curative test capability was fulfilled during this report period. A total of 87 experimental drugs were evaluated, results are summarized.

9. Human Peripheral Blood Lymphocytes in Adults from Thailand with Naturally Acquired Plasmodium falciparum and Plasmodium vivax

OBJECTIVE: To describe the proportions of T, B and Fc receptor bearing lymphocytes circulating in patients during infection with malaria and fourteen days after initiation of treatment.

BACKGROUND: Although interest in the cellular aspects of the immune response to malaria dates back many years, to the present there exists a void in our knowledge within this area. A study of the circulating lymphocytes in malaria patients was undertaken to provide basic data in this field. The technique used in this study employed sheep red cell rosettes to identify subclasses of lymphocytes (3). In addition, B lymphocytes were also characterized by staining for cell surface immunoglobulin (4).

METHODS: The heparinized blood of malaria patients infected with P. falciparum or P. vivax was processed by ficoll-hyque centrifugation to isolate circulating mononuclear cells (5). The percentage of circulating T lymphocytes was identified by rosette formation with sheep erythrocytes (E rosettes) at 5 minutes, 1 hour and 18 hours. The percentage of Fc receptor lymphocytes was determined by sheep red cell/ anti-sheep red cell (EA rosette) adherence. The proportions of circulating B lymphocytes were evaluated by sheep red cell/anti-sheep cell/ complement (EAC rosette) complexing and by lymphocyte staining with fluorescein labelled (FITC) anti-human immunoglobulin.

RESULTS: Lymphocytes from 49 malaria patients were studied in the course of this work. It was found that during infection there was a marked suppression in the percentage of circulating T lymphocytes as compared with control values (Table 1). These results were highly predictable regardless of incubation time. The proportion of Fc receptor cells were essentially unchanged. There was a clearcut elevation in the percentage of circulating B cells as shown by both the EAC and the FITC techniques. Overall leukocyte kinetics were also monitored by white cell counts and by differential counts.

Table 1 Lymphocyte Rosette and FITC Values of Malaria Patients

Lymphocyte Origin	E Rosette Cells (%)			EA Rosette Cells (%)	EAC Rosette Cells (%)	FITC Cells (%)
	5 minute incubation	1 hour incubation	18 hour incubation			
<u>Plasmodium <i>falciparum</i></u>	19-39	27-50	44-59	3-8	20-30	17-29
Range	29	41	52	5	25	24
Mean	$\pm$ 5.0	$\pm$ 4.7	$\pm$ 2.3	$\pm$ 1.7	$\pm$ 2.7	$\pm$ 7.1
<u>Plasmodium <i>vivax</i></u>	20-38	31-50	45-62	3-8	20-29	16-26
Range	29	41	54	5	24	22
Mean	$\pm$ 5.0	$\pm$ 4.8	$\pm$ 3.3	$\pm$ 1.5	$\pm$ 2.6	$\pm$ 2.7
Normal controls	36-52	49-63	60-66	2-7	14-17	9-18
Range	41	54	63	5	16	15
Mean	$\pm$ 4.0	$\pm$ 4.3	$\pm$ 1.6	$\pm$ 1.3	$\pm$ 1.0	$\pm$ 2.3

Table 2. Lymphocyte Rosette and FITC Values of Returning Cases

Lymphocyte Origin	E Rosette Cells (%)						EA Rosette Cells (%)			EAC Rosette Cells (%)			FITC Cells (%)			
	5 minute incubation		1 hour incubation		18 hour incubation		A		C		A		C		A	
	A <sup>+</sup>	C <sup>†</sup>	A	C	A	C	A	C	A	C	A	C	A	C	A	C
<u>Plasmodium</u> <u>falciparum</u>	Range	24-32	27-42	33-39	39-55	50-52	50-61	3-4	2-4	24-27	17-26	21-27	17-24			
	Mean	28	33	38	44	51	54	4	3	25	22	23	21			
	S.D.	± 4.0	± 7.9	± 5.0	± 9.3	± 1.0	± 5.8	± 0.6	± 1.0	± 1.5	± 4.6	± 3.2	± 3.6			
<u>Plasmodium</u> <u>vivax</u>	Range	21-30	21-34	32-42	31-45	54-56	51-60	4-6	3-5	20-24	21-26	19-26	18-24			
	Mean	25	28	37	40	55	55	5	4	21	23	21	21			
	S.D.	± 3.9	± 5.3	± 3.7	± 5.6	± 0.8	± 3.4	± 0.9	± 0.9	± 1.6	± 2.1	± 2.8	± 2.7			
Normal* Controls	Range	36-52		49-63		60-66		2-7		14-17		9-18				
	Mean	41		54		63		5		16		15				
	S.D.	± 4.0		± 4.3		± 1.6		± 1.3		± 1.0		± 2.3				

\* Same normal control group as indicated in Table 1

+ Values for active infection

† Values for convalescent stage

The lymphocytes of 5 returning vivax cases and 3 returning falciparum cases were studied by these techniques 14 days after the initiation of antimalarial therapy (Table 2). The data indicated a general pattern which was similar to that of the active infection. A manuscript on this work is in preparation. This project is complete.

#### 10. T Lymphocyte Subpopulations in Thai Malaria Patients

OBJECTIVE: To determine changes in the proportions of subpopulations of T lymphocytes of malaria patients in comparison with those of normal volunteers.

BACKGROUND: In a previous study conducted in this laboratory (6) it was determined that in human malaria patients there is depression in the percentage of circulating T lymphocytes as determined by the sheep red cell (E) technique. As an extension of this study we wished to determine whether these alterations involved only one subpopulations of lymphocytes or whether such changes were associated with all subpopulations of T cells. Current methodology allows for the isolation of lymphocyte subpopulations by physico-chemical technique (7, 8). It was thus decided to adopt these techniques to the study of subpopulations of malaria patient lymphocytes.

METHODS: In the course of these experiments the methodology of Glinski et al. (8) was employed with modification. Each sample of heparinized blood was centrifuged at 400 x g for 30 minutes in the cold. The resulting leukocyte rich layer was removed by Pasteur pipette and, after washing, the cells were standardized to a concentration of  $2 \times 10^6$  mononuclear cells/ml in Hank's balanced salt solution (HBSS). A 0.1 ml sample was assayed for the percentage of T lymphocytes forming E rosettes with sheep erythrocytes. The remainder of each aliquot of cell suspension was processed by discontinuous ficoll-hyphaque centrifugation. Samples were layered on a "column" of ficoll of graded concentration ranging from 30% ficoll at the bottom of the polycarbonate centrifuge tube to 9% at the top. Sample were centrifuged as before. Each centrifugation yielded seven cell fractions which were sequentially drawn off, washed and standardized in HBSS. Due to a scarcity of cells, the first 3 strata were pooled. Each aliquot was then standardized as before and studied for the percentage of E rosettes formed. All assays were made blind with the origin of the sample unknown to the technician. The percentages of E rosettes were cauculated

Table 1. Circulating T Lymphocyte Subpopulations in Malaria Patients

Assay No.	Percentage of E Rosettes After Ficoll-Hypaque Centrifugation											
	Unfractionated samples			Pooled subfractions			Separate subfractions					
	NC*	P†	‡	NC	P	NC	P	NC	P	NC	P	NC
1	ND‡	ND	6	5	7	7	12	10	9	11	6	7
2	ND	53	7	4	8	6	11	8	11	9	7	5
3	ND	55	4	6	6	6	10	10	11	10	7	6
4	58	54	6	5	6	6	11	10	10	10	6	6
5	60	52	5	5	7	6	9	10	11	11	5	6
6	59	54	5	5	6	7	10	10	11	10	6	7
7	60	55	6	5	9	6	11	10	10	10	7	6
8	59	55	6	5	8	6	11	11	10	10	7	5
9	60	52	6	4	8	6	11	10	11	10	7	6
10	61	53	6	6	8	7	10	10	11	11	7	8
Mean	59.7	53.7	5.7	5.0	7.3	6.3	10.5	9.9	10.5	10.2	6.5	6.2
S.D.	± 0.98	± 1.23	± 0.82	± 0.67	± 1.06	± 0.48	± 1.75	± 0.74	± 0.71	± 0.63	± 0.71	± 0.92
Relative decrease (%)	10.1	12.3			13.7		5.7		2.9		4.6	

\* NC = Normal control

† P = Patient

‡ ND = Not done

P. vivax patient, others are P. falciparum

from duplicate hemocytometer readings and the results averaged. In addition to determining the mean values for patients and normal controls, the relative decrease of the two populations was determined by the following formula:

$$\text{Relative decrease} = 100\% - \frac{\text{Patient mean}}{\text{Normal mean}} \times 100.$$

**RESULTS:** Table 1 summarizes the E rosette values for all T cell assays conducted. As in the previous studies cited (6) the T lymphocyte values of unfractionated samples were suppressed in malaria patients. The data indicated for each subfraction both a slight absolute suppression (mean values) and a relative decrease in the patient T cell subpopulations (mean values) in comparison with those from normal controls. There was also a suggestion that cells of lower density were more suppressed than those of greater density. A manuscript on this work has been submitted for publication. This project is complete.

#### 11. Production of Lymphokines and Chemotactic Activity of Malaria Patient Monocytes

**OBJECTIVE:** To develop assays for the production of lymphokines by malaria patient lymphocytes and for the measurement of chemotaxis of patient monocytes induced by such lymphokines.

**BACKGROUND:** The protective reaction to infection with malaria is notorious for its inefficiency. The immune response is characterized by excessive synthesis of immunoglobulin - most of which has no demonstrable anti-malarial activity. Although few investigations have been directed toward the cellular immunology of malaria, it has been reported that a concomitant state of immunosuppression stems from defective macrophage function (9).

It was reasoned that such defects might stem from defective responsiveness to monocyte chemotactic factors normally synthesized by lymphocytes. Such defects had been reported in other disease states such as the Wiskott-Aldrich syndrome (10) and chronic mucocutaneous candidiasis (11). As an extension of this rationale a preliminary battery of assays was conducted to test the migratory competence of monocytes across a membrane in response to lymphocyte associated chemotactic factors.

Table 1. Monocyte Responsiveness to Chemotactic Factors

Supernatant origin	Monocyte origin	Total # of runs	Chemotactic index (C.I.) range mean
E. coli	Patient	3	1.91 - 3.26 2.4
	Normal	4	0.87 - 3.68 1.8
Concanavalin A	Normal	4	0.19 - 1.52 1.1
	Normal	6	0.8 - 2.2 1.5
Phytohemagglutinin	Normal	3	1.0 - 2.7 1.6
	Normal	6	0.7 - 1.3 1.1
Purified Protein Derivative			
Malaria Antigen (human)	Normal	2	1.0 - 1.2 1.1
Malaria Antigen (simian)	Normal		

METHODS: The chemotactic test substances utilized were as follow: E. coli supernatant or factors (of lymphocyte origin) synthesized against human and simian malaria antigen or factors for the plant mitogens concanavalin A, phytohemagglutinin and purified protein derivative. For the chemotactic assays against E. coli supernatant  $2 \times 10^6/\text{ml}$  mononuclear cells from either normal Thai donors or malaria patients were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Controls for these assays were normal media. For the chemotactic assays against the malaria antigens and the mitogens  $2 \times 10^6/\text{ml}$  mononuclear cells from normal donors were incubated as above with the respective material over a wide range of concentrations. Controls for this series of assays incorporated the short term (non-incubation) exposure of mononuclear cells to each material prior to second-stage decanting of the supernatants for the chemotactic assay.

RESULTS: The results of chemotactic assays of normal and patient monocytes against E. coli supernatant and of normal monocytes against the above indicated materials are indicated in Table 1. The data suggests that there is demonstrable chemotactic activity by each parameter employed. It is equally clear that the chemotactic index in each case is much lower than had been hoped. While these values might be enhanced by purification and concentration techniques it is felt that these requirements vs. time and manpower are disproportionate to the relative value of such work. It is thus our intention to make our data available to other in-house investigators and to abort further pursuit of this work in favor of research with higher priority. This project should therefore be considered complete.

## 12. Circulating Lymphocytotoxic Factors in Malaria Patients

OBJECTIVE: To assay for substances in malarious plasma which are toxic for lymphocytes.

BACKGROUND: There have been several reports in the literature of plasma borne antibodies which are cytotoxic for autologous lymphocytes. Terasaki et al. reported cytotoxic factors in the plasma of patients infected with either systemic lupus erythematosus or rheumatoid arthritis (12). These findings were confirmed by the work of Michlmayr et al. (13) who indicated that both T lymphocytes and B lymphocytes were target cells although primarily the former were killed. Dettorarius et al. (14) extended these findings with their report of lymphocytotoxicity in patients infected with hepatitis. Earlier work in this laboratory (15) indicated that there is a marked

suppression in the proportion of circulating T lymphocytes during infection with malaria. It was reasoned that lymphocytotoxicity might be one mechanism underlying the observed phenomenon. We thus decided to screen patient plasma for cytotoxic activity under optimal conditions as reported by other workers.

METHODS: Cytotoxic assays were performed with the plasma of 42 patients infected with either P. falciparum and P. vivax. Methodology according to Terasaki (12) was employed with modification. Autologous control sera and target lymphocytes were obtained from normal volunteers. Lymphocytes isolated by ficoll gradient centrifugation were adjusted to a concentration of  $4 \times 10^3$  cells in 0.02 ml SBSS pH 7.3. Target cells were incubated with sera for 1/2 hour at 15°C at which time .02 ml of 1:5 dilution of complement was added; the incubation was continued for another 2 1/2 hours. Following incubation the percent of dead cells per 100 mononuclear cells were calculated by dye exclusion.

RESULTS: The results of these experiments indicated a general similarity in the percent of target cell death in the normal and test cultures of each series. In 5 test plasma cultures there was evidence of significant cell disruption. Cell death in these cultures ranged between 12% and 54% (mean 26%) as opposed to 7% in the single control culture. There was no evidence of cytotoxic activity in the plasma of 37 other malaria patients as compared with controls. Interpretation of the 5 positive plasma is difficult in that the target lymphocytes were provided by a donor who was later found infected with hepatitis. No activity was found in the plasma of these 5 patients when a follow-up assay was conducted against lymphocytes from the same donor or other donors. Due to the lack of firm evidence for cytotoxic activity in the plasma of these malaria patients work in this area has been aborted. This is a final report.

### 13. Mitogen Responsiveness of Lymphocytes from Patients Infected with Malaria

OBJECTIVE: To compare the responsiveness of malaria patient lymphocytes to stimulation by selected plant mitogens with responsiveness of normal lymphocytes.

BACKGROUND: The application of plant mitogen stimulation in the study of cellular immunology has been well documented. This

methodology has been most often utilized in the study of T lymphocytes but has also been employed in investigations of B lymphocyte activation (16). The salient feature of this approach is the assessment of mitogen mediated activation by blastogenesis or by enhanced uptake of radio labelled amino acids. These techniques were employed in the activation of malaria patient lymphocytes using the plant mitogens phytohemagglutinin (PHA), Concanavalin A (CON A), and Pokeweed mitogen (PWM).

**METHODS:** Mononuclear cells were isolated by ficoll hypaque centrifugation according to the methodology of Boyum (17). The cells were then washed in Selegmans balanced salt solution (SBSS) and further processed according to the methodology of Chess et al. with modification (18). Each aliquot of cells was adjusted to  $1.5 \times 10^6$  cells/ml in RPMI 1640 media in flat bottom microtiter plates. Each assay was conducted in a set of 10 wells. Mitogens were added in the following concentrations per microtiter well: PHA, 5 ug; CON A, 2 ug; PWM, 25 ug. Cultures were incubated for 72 hours in 5% CO<sub>2</sub> and were pulsed with 0.4 uCi <sup>3</sup>H-thymidine for 24 hours. Samples were harvested with a multi-sample harvesting machine and washed with distilled water. Dried filter papers discs were then transferred to scintillation vials and suspended in Hydromix before counting in a Hewlett-Packard beta counter. The mean value for the counts per minute (CPM) and the standard deviation of the mean were determined. The stimulation index (S.I.) was determined for each sample. The S.I. was calculated with the following formula: S.I. = mean test CPM/mean control CPM.

**RESULTS:** Table 1 summarizes the PHA response of patient lymphocytes as compared with that of uninfected volunteers. The response of patient lymphocytes is not significantly different from that of the normal controls (mean values 60,954 vs 59,897 CPM respectively). The S.I. results suggest a lower PHA stimulation of patient lymphocytes. These results are, however, somewhat misleading since the S.I. is highly influenced by variation in the unstimulated control cultures. In a few assays the CPM in the control cultures were excessively high and the corrected CPM therefore gives more reliable data. Responsiveness of patient lymphocytes to stimulation with CON A (Table 2) was significantly lower than normal lymphocytes. Mean CPM values were 23,260 vrs 28,880 respectively while values for the S.I.'s were 38.1 and 43.0. The PWM response (Table 3) indicates similar results for patients and controls. The mean CPM values were 29,548 for patients and 28,612 for controls while the S.I.'s were 44.4 and 42.2. The overall conclusions from

Table 1: PHA Response of Human Lymphocytes Normal vs Patients with Malaria

Type of Infection	CPM		S.D.		S.I.	
	Patient	Control	Patient	Control	Patient	Control
P.V.	80318.67	47164.67	4231.40	5888.81	101.56	134.73
P.F.	63722.94	56851.33	10101.02	6167.80	62.76	36.26
P.V.	60211.50	70402.00	4787.71	18530.64	63.82	76.49
P.V.	81716.67	50268.07	8292.28	4197.17	60.69	51.69
P.V.	74686.34	44581.66	5788.40	2691.16	62.23	56.73
P.V.	53735.50	88429.34	8424.37	13515.63	43.45	198.53
P.V.	60946.83	64727.85	8676.35	8348.38	40.80	56.44
P.F.	65889.83	58501.66	3554.21	9310.35	85.09	172.05
P.F.	52528.40	49503.40	4525.07	2711.54	61.03	95.83
P.F.	47506.08	48450.66	2734.23	4532.59	91.53	71.21
P.V.	71673.00	51913.83	4202.38	3039.66	158.26	68.24
P.V.	59595.06	66697.40	8646.78	5089.15	145.97	38.30
P.F.	44783.17	60399.33	2379.39	3346.25	88.07	106.16
P.V.	47700.15	69055.83	3014.45	10162.36	141.45	119.95
P.F.	49309.60	71516.50	3462.36	7835.34	56.59	117.22
Mean	60954.91	59879.66	5521.36	7024.45	84.22	93.32

Table 2: Con-A Response of Human Lymphocytes Normals vs. Patients with Malaria

Type of Infection	CPM		S.D.		S.I.	
	Patient	Control	Patient	Control	Patient	Control
P.V.	21437	16993.61	3975.83	5376.43	27.84	49.18
P.F.	14648.34	32894.67	1590.26	6845.42	15.19	21.40
P.V.	23202.50	40032.67	4211.39	4554.16	25.21	43.93
P.V.	15116.00	28399.23	2886.33	4616.20	12.04	29.63
P.V.	34532.80	20278.66	5279.55	1784.14	29.31	26.09
P.V.	23545.67	27345.17	4163.42	4632.56	19.60	62.08
P.V.	34098.17	41888.26	6380.89	6321.78	23.26	36.88
P.F.	28210.50	24325.66	2012.72	4056.54	37.0	72.12
P.F.	24900.17	31101.33	3900.19	6767.93	29.45	60.58
P.F.	28724.91	20315.83	2946.31	4778.17	55.74	30.44
P.V.	34163.75	238889.25	2919.29	3687.85	75.96	31.94
P.V.	37352.40	32336.53	2541.00	1608.86	91.86	19.08
P.F.	19978.17	33591.67	1358.22	3641.95	39.84	59.48
P.V.	20168.06	29772.66	3987.32	5592.75	60.38	52.28
P.F.	24912.50	30037.83	2702.04	5082.04	29.08	49.81
Mean	23260.37	28880.20	3390.31	4623.11	38.11	42.99

Table 3: PWM Response of Human Lymphocytes Normals vs Patients with Malaria

Type of Infection	Patient	CPM Control	Patient	S.D. Control	Patient	S.I. Control
P.V.	25034.0	14201.74	3217.01	4867.75	32.34	41.27
P.F.	30940.34	24425.50	2669.25	2832.52	30.99	16.15
P.V.	29286.83	57325.75	3706.30	10466.97	31.36	62.47
P.V.	14972.00	22794.73	2049.15	1938.88	11.93	23.98
P.V.	30310.51	23293.16	3828.76	1906.73	25.85	29.82
P.V.	33154.17	17125.50	4684.42	2571.13	27.19	39.25
P.V.	37537.67	55397.26	6696.60	5724.47	25.51	48.45
P.F.	32463.83	31008.00	2060.85	3082.87	42.43	91.66
P.F.	16774.75	22359.50	1022.40	3489.70	20.17	43.88
P.F.	27369.58	19456.33	3328.38	1529.76	53.15	29.19
P.V.	45750.00	23399.83	4132.98	2637.83	101.38	31.31
P.V.	46545.90	25248.53	4056.37	1949.80	116.61	15.12
P.F.	21192.50	29701.33	3357.35	2091.85	42.20	52.71
P.V.	24084.06	25357.50	4125.27	5947.58	71.91	44.75
P.F.	27812.16	38099.83	4450.02	4153.85	32.35	62.91
Mean	29548.55	28612.23	3559.00	3679.44	44.37	42.19

this study are that responsiveness of patient lymphocytes to PHA and PWM are normal. The responsiveness to CON A appears slightly depressed and may be attributed to a missing T cell population as has been reported elsewhere. A manuscript concerning this work is in preparation. This project is complete.

14. Malaria Patient Lymphokine Mediated Macrophage Activation as Demonstrated by Enhanced Adherence and Protein Synthesis

OBJECTIVE: To develop an assay to describe lymphokine mediated activation of macrophages by the parameters of increased macrophage adherence and protein synthesis (lowry).

BACKGROUND: Data by Criswell and her colleagues (19) confirms earlier notions that humoral factors, possibly other than antibody, may activate macrophages in the destruction of the malaria parasite. Although research in this area is just now being started by mafriologists, workers in other field have provided such insight through related systems (20,21). This report summarizes preliminary data in an effort to apply the theory and technology of these studies to investigations of the human response to malaria.

METHODS: The assays conducted were accomplished according to the methodology of Nathan and his colleagues (22). In synthesising lymphokines,  $2 \times 10^6$  patient mononuclear cells were incubated with P. vivax antigen either full strength or diluted 1:2 for 48 hours. Cultures were then centrifuged and supernatants frozen until required. Control supernatants were prepared by incubating patient cells with media only, adding antigen immediately prior the centrifugation step. For the adherence assay in the presence of lymphocytes supernatants were incubated with normal mononuclear cells for 3 days. After decanting the supernatants monolayers were treated with NaOH and Lowry protein concentrations were determined. An adherence assay was also made in the absence of lymphocytes; for this all steps outlined above were accomplished except that the supernatant containing non-adherent lymphocytes was decanted after 2 hours followed by washing in hepes buffer before the 3 day incubation was begun.

RESULTS: Table 1 summarizes the results of macrophage adherence with lymphocytes present. The test series shows an overall mean value of 77 ug/ml protein vs 56 ug for the control group. Data in Table 2 suggests a similar pattern with mean values of 59

Table 1. Lowry Protein Values (ug/ml) - Lymphocytes Present

Specimen No.	Test		Control
	High/Low Initial Ag Conc.	Mean	
1	119.5 101.1	110.3	61.8
2	82.5 80.3	81.4	95.8
3	65.6 86.2	75.9	47.9
4	39.0 42.0	40.5	18.0
Overall Mean		77.0	55.9

Table 2. Lowry Protein Values (ug/ml) - Lymphocytes Absent

Specimen No.	Test		Control
	High/Low Initial Ag Conc.	Mean	
1	79.5 85	82.3	33.3
2	74.4 57.5	66.0	33.2
3	82.5 60.4	71.5	45.0
4	12.0 21.0	16.5	25.5
Overall Mean		59.0	34.3

and 34 for test and control groups respectively. The preliminary data in both of these assays suggests an enhancement in macrophage activation mediated by antigen stimulated lymphokine synthesis. These assays seem encouraging and serve as the basis for continuing studies.

#### 15. Glucosamine Uptake in Malaria Activated Human Macrophages

OBJECTIVE: To develop an assay to describe the activation of monocytes through interaction with malarious lymphocytes as determined by increased uptake of radiolabelled glucosamine ( $^{14}\text{C}$  glu).

BACKGROUND: The mechanisms of host response to malaria infection at the cellular level are poorly understood at present. Findings by other workers have provided a basis for pursuing basic investigations in this area (23). It was reasoned that techniques could be developed to evaluate activation of mononuclear cells through radioimmunotest. This report describes preliminary results from these studies.

METHODS: The assays conducted were modifications of the methodology of Hammond and Dvorak (24). Mononuclear cells were isolated by ficoll-hypaque centrifugation and adjusted to a concentration of  $1.5 \times 10^6$  monocytes per culture. Test antigens consisted of either pooled *P. vivax* merozoite or sporozoite antigens. Positive controls consisted of cells and PHA mitogen, negative controls were cells and RPMI 1640 media only. Each cell culture was incubated in 5%  $\text{CO}_2$  for 72 hours. The cultures were then pulsed with 5 uCi of glucosamine with continued incubation for 6 hours. Each monolayer was washed with cold Hank's containing non-radiolabelled glucosamine. Vials were drained and frozen until required. For the conduct of the assay, the contents of each vial were treated with cold 10% TCA containing calf serum and stored at  $4^\circ\text{C}$  1 hour. After centrifugation the supernatant was decanted and Hydromix was added to precipitate the radio-labelled proteins. Counts were performed in a Hewlett-Packard beta counter.

RESULTS: The results of a single assay completed are unremarkable. The positive (PHA) control indicated stimulation as expected but the antigen - test values resembled those of the negative control. The development of this assay continues.

16. Transformation of Patient Lymphocytes by Selected Malaria Antigens

OBJECTIVE: To develop preliminary methodology for the direct stimulation of malaria patient lymphocytes by erythrocyte and sporocyte antigens as a basis for more definitive studies.

BACKGROUND: The availability of assays with sufficient specificity and sensitivity presents a major barrier to the understanding of host immune response mechanisms in malaria. There are several recent reports in the literature which may assist in the solution of this problem. Of special interest are papers dealing with the in vitro stimulation of sensitized lymphocytes by malaria antigen employing either rodent (25,26) or human material (27). This report summarizes the results of pilot studies employing similar techniques with material from Thai malaria patients. These assays are critical to the success of more definitive heterogeneity studies using cells and antigens from different geographic regions.

METHODS: Isolated mononuclear leukocytes were cultured in modified RPMI 1640 media according to the methodology of MacDermott et al. (28). Assays for lymphocytes from falciparum (P.F.) infections used lymphocytes from P.F. patients and P.F. antigen extracted from infected chimpanzee erythrocytes. Assays for vivax infections (P.V.) were done with lymphocytes from vivax infections and P.V. sporozoites. Controls consisted of cells and media not containing antigen. Cell suspensions were incubated with antigen for 6 days at 37°C in 5% CO<sub>2</sub>. Culturers were then pulsed with 0.4 uCi <sup>3</sup>H thymidine and further incubated for 24 hours. The cells were then isolated and washed by a multiple automated sample harvester (MASH). After drying, individual filter pads containing the cells were placed in scintillation vials containing hydromix and were counted (CPM) in a Hewlett-Packard beta scintillation counter.

RESULTS: The findings of 8 preliminary assays are summarized in Tables 1 and 2. The stimulation ratios (SR) of patient lymphocytes vs controls were calculated by the following formula:

$$SR = \frac{CPM\ test}{CPM\ control}$$

Table 1 illustrates the values of 5 assays conducted in P.F. system. The SR range was 1.5-3.7 (mean 2.4). The SR values of 3 assays thus far conducted with the P.V. system are indicated in Table 2. Here the range was 1.4-2.3 (mean 2.0). With both approaches it is clear that further work is necessary to develop the assay. Modifications are underway which involve both cell and radioisotopes concentrations are incubation time. This project continues.

Table 1. Stimulation Ratios: P. falciparum Antigen  
 (Chimpanzee/RBC) x human falciparum Lymphocytes

Assay #	Stimulation Ratio
1	2.2
2	2.3
3	3.7
4	1.5
5	2.2
Range	1.5 - 3.7
Mean	2.4

Table 2. Stimulation Ratios: P. vivax Antigen  
 (human/sporozoite) x human vivax Lymphocytes

Assay #	Stimulation Ratio
1	1.4
2	2.3
3	2.3
Range	1.4 - 2.3
Mean	2.0

17. Synthesis of Lymphocyte Blastogenic Factor by Sensitized Lymphocytes from Malaria Patients

OBJECTIVE: To develop preliminary methodology for the activation of normal lymphocytes by lymphokines induced by malaria antigens as a basis for more definitive studies.

BACKGROUND: While it may be assumed that the mechanisms of immunologic activation in malaria parallel those of better studied systems, at present few studies have been accomplished to support or refute this assumption. One feasible parameter of exploration is the application of lymphokines elicited through the interaction of antigens with specifically sensitized lymphocytes. There is a considerable body of evidence that non-sensitized lymphocytes undergo blast transformation and incorporate  $^{3}\text{H}$  thymidine when cultured in the presence of supernatants from such stimulated lymphocytes (29,30). The synthesis of the blastogenic factor(s) is dependent upon T lymphocytes as opposed to B lymphocytes and is considered to be a manifestation of cell mediated immunity. The studies reported here are preliminary to more definitive investigations of activation mechanisms in malaria.

METHODS: Mononuclear leukocytes from *P. falciparum* patients were isolated by ficoll-hyphaque centrifugation. The cells were incubated with falciparum antigen from infected chimpanzee erythrocytes for 48 hours at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . Following centrifugation supernatants were frozen until required. Control preparations were supernatants exposed to antigen immediately before centrifugation. For the remainder of the assay non-sensitized lymphocytes were incubated with the supernatants for 6 days. Cultures were then pulsed with 0.4 uCi  $^{3}\text{H}$  thymidine before incubating another 24 hours. The cells were isolated and washed in a multiple automated sample harvester (MASH). After drying on filter pads the cellular material was placed in a scintillation vial containing hydromix. Counts for radioactivity (CPM) were conducted on a Hewlett-Packard beta counter.

RESULTS: Table 1 summarizes the results from assays on the blastogenic activity of supernatants from 3 malaria patients. Although the differences between test and control values are small, they are within the range of results from earlier workers using other systems (30). It is clear, however, that further standardization toward optimal conditions is in order. Alterations of cell concentration, type and concentration of antigen and incubation time are planned. This project continues.

Table 1. Results from Preliminary Studies on Blastogenic Activity from Malaria Patient Lymphokines

Assay #	Test (CPM)	Control (CPM)
1	1332	694
2	1405	1115
3	911	439
Range	911 - 1332	439 - 1115
Mean	1216	749

## 18. In vitro Gametogony of Plasmodium falciparum

OBJECTIVE: To establish an in vitro technique for the maintenance and replication of sexual erythrocytic forms of Plasmodium falciparum.

BACKGROUND: Although the sexual forms of plasmodia do not cause clinical symptoms, their existence could prevent the eradication of malaria in areas endemic for this disease. Some antimalarials result in an elevation in gametocytes during treatment (31) and the gametocidal effectiveness of drugs therefore needs to be tested. An in vitro test system would greatly facilitate such studies. To this end earlier techniques were developed for the in vitro culture of erythrocytic asexual forms of P. falciparum parasites (32). The culture techniques also proved well suited for the study of antimalarials effectiveness against plasmodial forms by the parameters of morphology and radiotechnique (33). This report summarizes attempts to improve in vitro culture technique for the maintenance and replication of sexual forms of Plasmodium falciparum.

METHODS: Heparinized blood from malarious patients was twice washed in normal saline. After final centrifugation the packed cells were resuspended in medium T-199 and parasitemia adjusted to 0.5% by adding washed cross matched erythrocytes. Heat inactivated pooled AB serum was added to the cell suspension to a final concentration of 40% (v/v). The parasitized blood was incubated at 37°C with addition of media every 48 hours and fresh erythrocytes were added just prior to anticipated merozoite reinvasion. Cultures were monitored by Giemsa stained blood smear.

RESULTS: Blood specimens collected from patients at the Somdej Sri Racha Hospital, Cholburi were cultured and compared with those of earlier work. It was found that gametocytes developed only in cultures of specimens collected at times of low level malaria transmission. During these studies gametocyte replication was low and inconsistent. Among 30 cultures performed 1 culture indicated replicating gametocytes. This positive culture developed at 96 hours incubation. An attempt to infect mosquitoes with the cultured gametocytes proved negative. In view of the paucity of new data from these studied it was decided to discontinue further work in this study area in favor of projects with higher priority. This is a final report.

## 19. Ecology of Malaria in Northern Thailand

**OBJECTIVE:** To investigate the reappearance of malaria in the Chiangmai Valley and other areas of Northern Thailand.

**BACKGROUND:** The Chiangmai Valley was site of the first malaria eradication program (1949-1950) in Thailand, and the valley floor has been considered free of all but imported malaria cases for the past twenty-five years. Malaria has persisted, however, in the mountains and foothills surrounding Chiangmai Valley, and during the past three years the Malaria Eradication Project has reported the occurrence of apparently indigenous cases of malaria among residents of the valley floor. Entomological surveys carried out by our lab in Chiangmai province between 1962 and 1970 recorded the presence of Anopheles minimus, the principal vector of malaria in Chiangmai Valley in past years, in Doi Saket, Mae Rim, Muang, Sankampaeng and Saraphi Districts, all located on the floor or at the edge of the valley. Other potential vectors such as Anopheles balabacensis and Anopheles maculatus have been found in these areas. It is apparent that, despite many years of eradication efforts in and around the Chiangmai Valley, populations of malaria vectors are still present at its periphery and may be extending their ranges onto the valley floor once again.

**METHODS:** From August 1976 a surveillance of anopheline populations was carried out in Doi Saket, Hang Dong, and Mae Rim Districts on a monthly basis until September 1977. Three rural villages in each district were surveyed, with one of the villages being located in the foothills, one on the valley floor, and the other situated in between. Human population in the nine villages is 3,580.

Battery powered CDC light traps together with a CO<sub>2</sub> attractant (dry ice) were used to monitor adult anophelines. In each area on a particular night, twelve traps were placed outside of houses and six were placed inside. In one month each of the nine villages was visited three times, with traps being set in the evening and being picked up in the morning. Light trap collections were sorted according to mosquito species and all suspected vectors were dissected and examined for malaria parasites. Recently engorged females were preserved for precipitin tests. Some indoor and outdoor human bait collections were made periodically with the adults being processed the same way as those caught in light traps.

Table 1. Anopheles spp. Collected in CDC Light Traps (with CO<sub>2</sub>) - Chiangmai, August 1976 - August 1977

District	A	S	O	N	D	J	F	M	A	M	J	J	A	Total
Amphor Doi Saket	367	414	149	116	15	26	57	79	79	96	101	415	131	2,045
Amphor Mae Rim	175	372	665	125	145	27	143	130	176	295	560	556	242	3,611
Amphor Hang Dong	314	143	86	61	10	21	139	116	11	90	27	318	60	1,396
Total	856	929	900	302	170	74	339	325	266	481	688	1289	433	7,052

Larval collections were made at all the villages to detect anopheline breeding sites. Surveys were conducted each month to measure fluctuation of anopheline populations.

RESULTS: During the past year the National Malaria Eradication Project reported a total of six cases of malaria in all three study villages in Amphor Doi Saket, one case in the Amphor Mae Rim foothill village, and three cases in the Amphor Hang Dong village near the foothills. They applied DDT to houses in only four of the nine study villages, the others having had no control since 1963.

A total of 7,052 Anopheles mosquitoes were collected in CDC light traps (with CO<sub>2</sub>) in the three Chiangmai districts (Table 1). As expected, anopheline populations were highest during the warm wet months. Collections in December and January were reduced greatly due primarily to colder temperatures in those months. Anopheles kochi, nivipes, and vagus were caught in the greatest numbers throughout the year.

Table 2 lists the six species considered by the Malaria Project people in Thailand to be possible potential malaria vectors, and the total number of each collected.

Anopheles aconitus, maculatus, and minimus collections by month are shown in Figure 1. Anopheles aconitus was caught most frequently, particularly in October and December. Collections of Anopheles minimus were very low from October to May. Figure 2 shows that Anopheles campestris was caught primarily in October and February. The other two species were captured in any numbers only in August, 1976.

All six potential vector species were dissected and the ovaries examined. Individuals found to be parous were further dissected and examined for malaria parasites. Of eleven hundred females dissected, 49 percent were found to be parous. None of these had oocysts on their guts or sporozoites in their salivary glands.

Of the 292 Anopheles minimus caught in light traps, one third were in traps which had been placed inside houses. Some of these had recently taken a bloodmeal. This species was found most frequently in Amphor Mae Rim, especially during 1977 (Figure 3). Larvae of this species were collected in all three districts and were found in two out of the three villages located on the valley floor. Adults were found in every village but one.

Bloodmeal analysis of engorged females is not yet complete but all other aspects of this study are.

Table 2. Potential Malaria Vectors Collected in CDC  
Light Traps (with CO<sub>2</sub>) Chiangmai Valley,  
August 1976-August 1977.

Species	Total Number
<u>Anopheles aconitus</u>	722
<u>Anopheles campestris</u>	125
<u>Anopheles culicifacies</u>	58
<u>Anopheles jeyporiensis</u>	58
<u>Anopheles maculatus</u>	258
<u>Anopheles minimus</u>	292

Fig. I CDC Light Trap (with CO<sub>2</sub>) Collections — Chiang Mai.

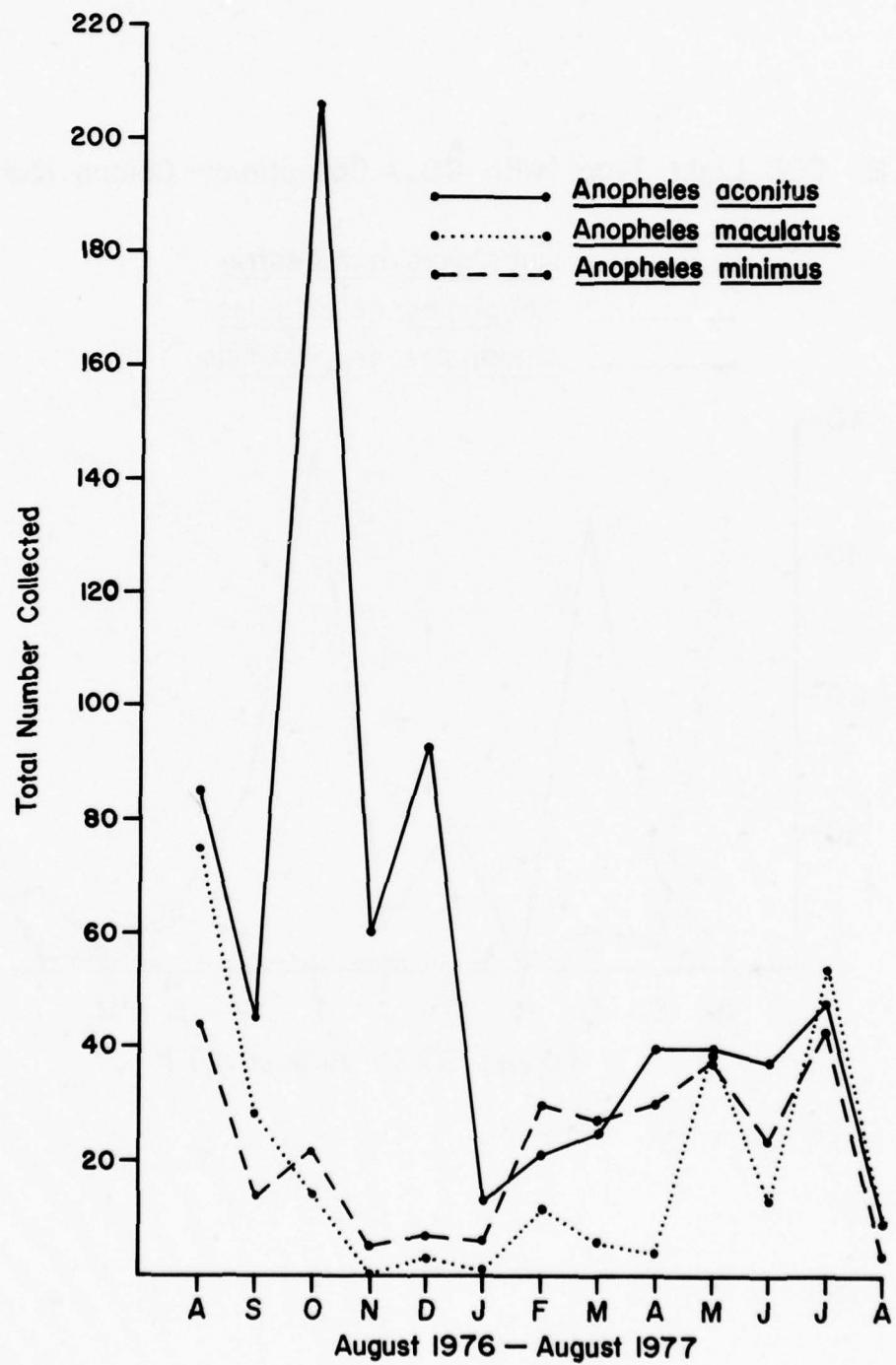


Fig. 2 CDC Light Trap (with CO<sub>2</sub>) Collections - Chiang Mai.

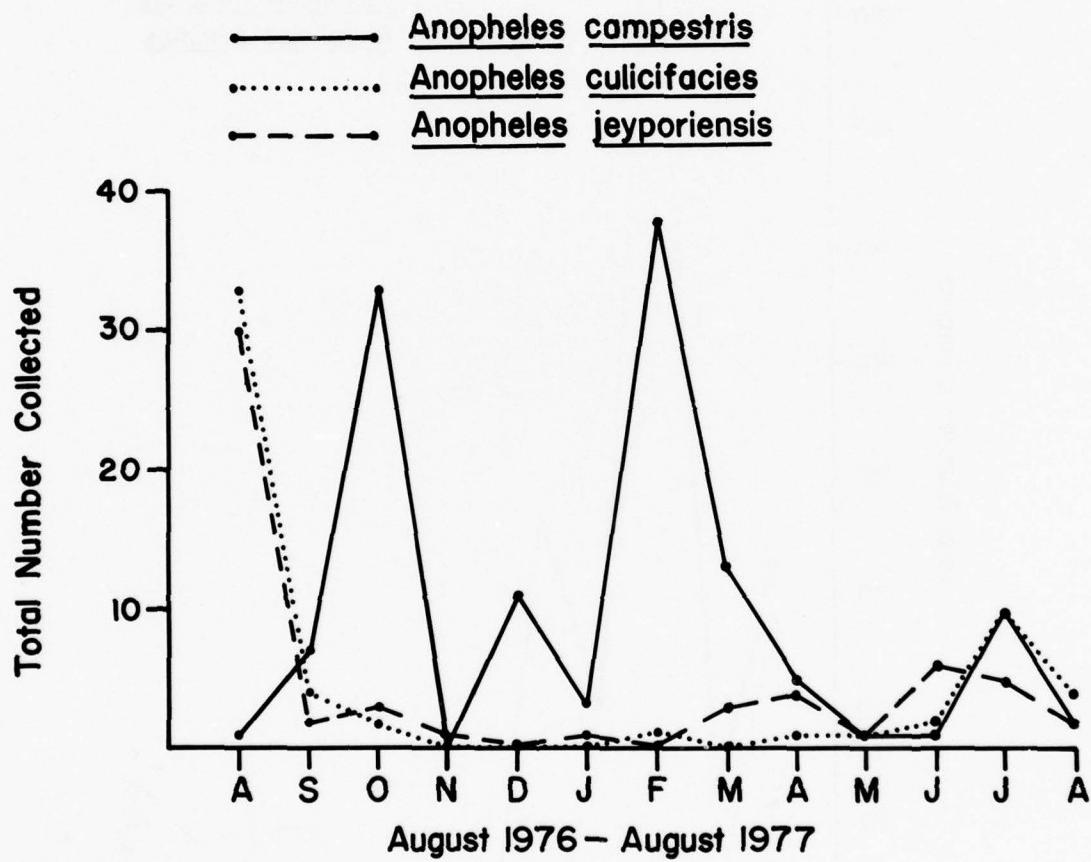
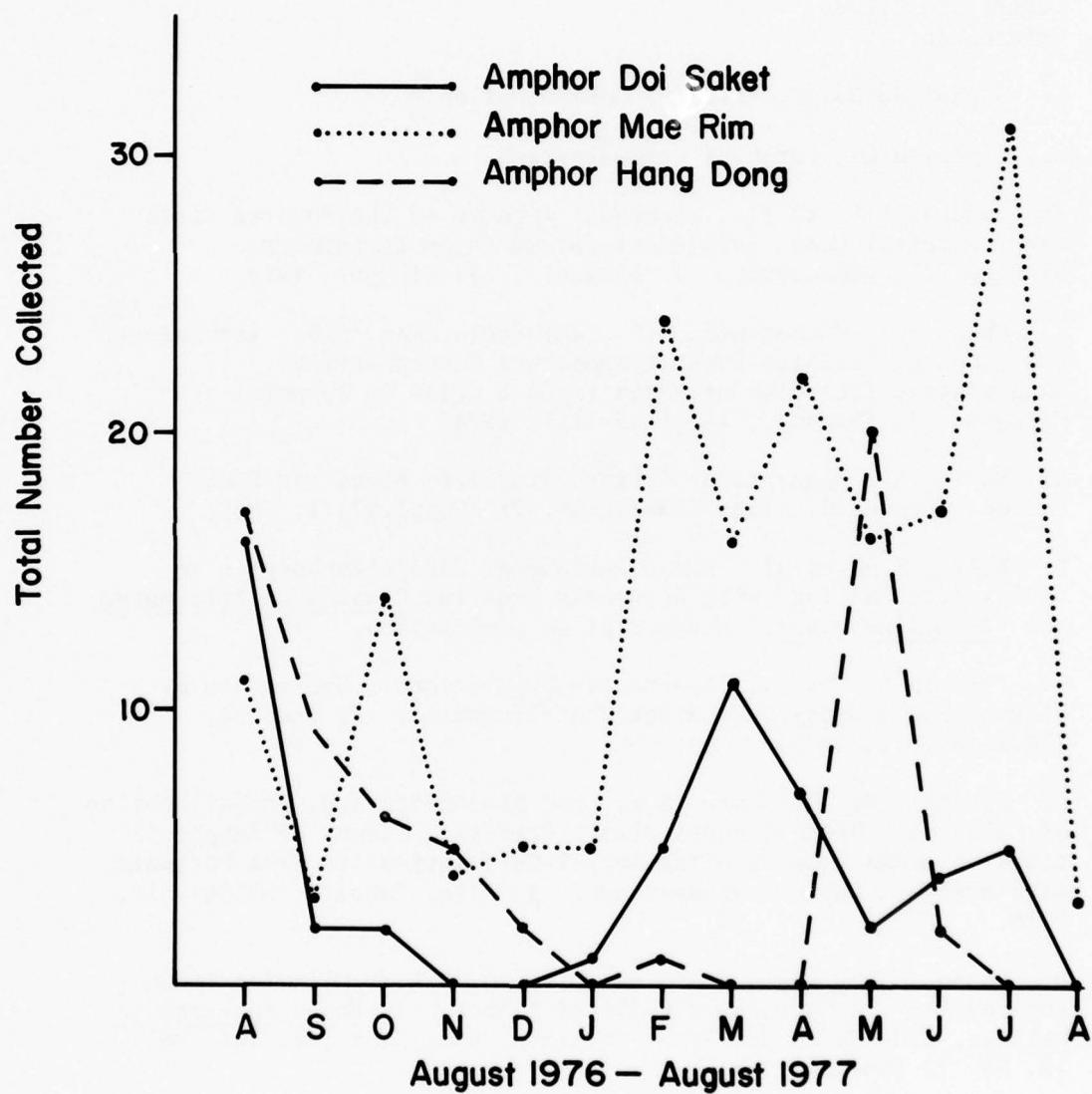


Fig. 3 CDC Light Trap (with CO<sub>2</sub>) Collections of Anopheles minimus- Chiang Mai.



Project 3M762770A803 MALARIA PROPHYLAXIS

Task 00, Malaria Investigations

Work Unit 089 Field studies on drug resistant malaria (SEATO)

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Project 3E762771A804

MILITARY PSYCHIATRY

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>2</sup>	2. DATE OF SUMMARY <sup>3</sup>	REPORT CONTROL SYMBOL
1. DATE PREV SUMRY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SECY <sup>4</sup> U	6. WORK SECURITY <sup>5</sup> U	DA OC 6450	77 10 01	DD-DR&E(AR)636
10. NO./CODES <sup>6</sup>				7. REGRADING <sup>7</sup>	8. DISCHG INSTRN <sup>8</sup> NA NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
PROGRAM ELEMENT				PROJECT NUMBER 3E762771A804	TASK AREA NUMBER 00	WORK UNIT NUMBER 041
a. PRIMARY b. CONTRIBUTING c. <del>CONFIDENTIAL</del> CARDS 114F						
11. TITLE (Precede with Security Classification Code) <sup>9</sup> (U) Behavioral Variables in Autonomic Function and Disease in Military Personnel						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>10</sup> 013400 Psychology 012900 Physiology 016200 Stress Physiology 02500 Clinical Medicine						
13. START DATE 76 07	14. ESTIMATED COMPLETION DATE CONT		15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House		
17. CONTRACT/GRAANT				18. RESOURCES ESTIMATE	19. PROFESSIONAL MAN YRS	20. FUNDS (In thousands)
a. DATES/EFFECTIVE: N/A				FISCAL YEAR 77 CURRENT	2	149
b. NUMBER: c. TYPE: d. AMOUNT: e. KIND OF AWARD:				FISCAL YEAR 78	2	139
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION		
NAME: Walter Reed Army Institute of Research Washington, D.C. 20012				NAME: Walter Reed Army Institute of Research Div of Neuropsychiatry		
ADDRESS: 				ADDRESS: Washington, D.C. 20012		
RESPONSIBLE INDIVIDUAL NAME: Rapmund, COL G. TELEPHONE: (202) 576-3551				PRINCIPAL INVESTIGATOR (Puruse SBN if U.S. Academic Institution) NAME: Hursh, CPT S.R. TELEPHONE: (202) 576-2483 SOCIAL SECURITY ACCOUNT NUMBER: <span style="background-color: black; color: black;">[REDACTED]</span>		
21. GENERAL USE Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS NAME: Faden, MAJ A.I. NAME: Cuthbert, CPT B.N.		
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Physiology; (U) Emotions; (U) Stress; (U) Autonomic Function; (U) Military Psychiatry; (U) Conditioning						
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Puruse individual paragraphs identified by number. Precede each with Security Classification Code) 23. (U) This is a multidisciplinary effort addressing the development and use of laboratory models to define and describe the organ system responses and disease states caused by stressors in the military environment.						
24. (U) The techniques of operant and respondent conditioning will be employed in the production of models of both phasic and chronic psychological and emotional stress. Cardiovascular function will be monitored by means of chronic indwelling catheters, which will also permit periodic sampling for assessment of hematological and hormonal effects. Electrophysiological measurements of central and autonomic responsiveness will provide both a means of fully interpreting similar data collected in field or laboratory studies with human volunteers, and a fertile source of hypotheses to identify potentially effective measures for prevention and treatment of this class of disorders in military personnel.						
25. (U) 76 10 - 77 09 Progress for the initial year: Studies of operant-respondent conditioning interactions with concomitant measurements of cardiovascular responses showed that environmental conditions can generate both increases in blood pressure with increases in heart rate and increases in blood pressure with similar decreases in heart rate. Tasks to obtain food demanding constant, intensive attention can generate larger increases in cardiovascular response than less demanding tasks to avoid an aversive stimulus, an important new dimension of stress. Methods were developed for measuring evoked potentials, heart rate, blood pressure, and blood flow in chronically aroused primates. Autonomic system studies have discovered an intraspinal sympathetic pre-ganglionic pathway that shares in the control of heart rate and cardiac contractility. For technical report see Walter Reed Army Institute of Research Annual Progress Report, <span style="background-color: black; color: black;">[REDACTED]</span> 1446						

DD FORM 1498  
1 MAR 68

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3E762771A804 MILITARY PSYCHIATRY

Work Unit 041 Behavioral variables in autonomic function and disease  
in military personnel

Investigators.

Principal: Hursh, CPT S.R.

Associate: Faden, MAJ A.I. and Cuthbert, CPT B.N.

Description.

Work within this unit has been directed toward the development of techniques of operant and respondent conditioning which can be employed as models of both phasic and chronic psychological and emotional stress and the application of the most current technologies for the monitoring of concomitant autonomic responses. The methodologies of both psychology and neurophysiology have been wedded in multi-level studies of behavioral, systemic, and neural changes. The first category of studies has focused on the control of autonomic responses by complex environment conditions requiring either intense and extended behavioral involvement or exposure to noxious and stressful conditions. The second category of studies has focused on the neural pathways and interrelationships which probably mediate the autonomic responses to these conditions.

BEHAVIORAL VARIABLES IN AUTONOMIC FUNCTION

Interactions between operant and respondent conditioning: This ongoing experiment was designed to study the effects of respondent conditioned stimuli imposed upon operant baselines. Among the goals of this study are the documentation of the physiological and behavioral effects of unavoidable appetitive and aversive signals during different kinds of tasks, and an examination of the relationships between operant and respondent conditioning as revealed by the results when the two are present simultaneously.

The data for the current year support previous indications that the nature of these relationships is more complex than previously thought. In particular, there appears to be an effect of the order in which the unconditioned stimuli are added to the experiment. This effect is most noticeable in the DRL experiment, in which a response must follow the previous response by at least 60 seconds in order to produce food. When a single tone becomes a conditioned stimulus (CS) by continued pairings of tone and shock during DRL performance, the result is an increase in blood pressure and a marked decrease in heart rate (HR), especially in the later parts of the CS. This pattern was seen in several monkeys run previously and looks like it will again be replicated in a currently running monkey who has just entered this phase of the experiment. When simultaneous respondent conditioning for food and shock is initiated with two different CS's, the blood pressure response is again an

increase; however, the heart rate response lacks the strong decelerative component. An earlier monkey showed marked acceleration of HR during the CS, and another subject who has just completed this phase displayed an inconsistent response which fluctuated back and forth between acceleration and deceleration over days. The response to the food CS has been generally small in both paradigms.

An order difference also appears in the experiment in which a CS superimposed upon Sidman avoidance signals free food. When the CS is introduced into the avoidance session only after conditioning is well established outside of avoidance periods, the result tends to be a decrease in response during the CS. However, in one monkey conditioning was initiated in the avoidance sessions without prior food-reinforced extra-avoidance trials; in this case, response rate increased during the CS.

These findings suggest two points. First, the interaction effects in these paradigms are evidently quite complex; while this does not necessarily mean that there are no consistent relationships between respondent and operant conditioning, it does imply that a careful experimental analysis must be performed in order to delineate precisely the controlling variables. Second, rather than conceptualizing simple decreases and increases in arousal in these situations, it is evident that the total organization of the cardiovascular system's response must be considered. Quite different cardiovascular substrates are obviously present when similar increases in blood pressure generate massive increases in heart rate in one instance, and equally large decreases in another. The nature and meaning of these response patterns must be considered in each case, and hopefully related to the corresponding behavioral results. Work is continuing on this experiment to replicate and clarify these findings.

Somewhat orthogonal information has been gained from these experiments by measuring tonic levels of physiological activity before and during experimental sessions. The monkeys run this year have uniformly supported the pattern described in last year's report that DRL sessions generate larger and more persisting increases in HR and blood pressure than Sidman avoidance periods. This contradicts the generally accepted notion that avoidance performance is more stressful than food-motivated responding. Consideration of the DRL schedule has led to the hypothesis that a task requiring absolutely constant, unceasing attention is very stressful no matter what the reinforcer. An experiment will be performed in the coming year to test this hypothesis by studying two levels of continuous attention during both negative and positive reinforcement; it is predicted that high attention demand will be more stressful overall than low attention demand, with negative reinforcement more stressful than a positively reinforced task. Stress will be operationally measured by recorded levels of physiological activity.

Evoked potentials in the monkey: A single pilot monkey was conducted to study the feasibility of recording EEGs in a chair-restrained preparation in the laboratory. This procedure had to be terminated somewhat prematurely when the monkey experienced renal failure and had to be sacrificed. However, some results were obtained. The task was a

discriminated reaction time task. Three tones of different frequencies were presented at varying intervals averaging 20 seconds. The higher two tones signaled reinforcement: response within a specified time (typically 350 milliseconds) following either of these tones resulted in delivery of a food pellet. A response to the lowest-frequency tone caused a one-minute time-out from the task. The ultimate goal was to make the lowest and middle tones close together in frequency of the highest tone different enough to be very easily discriminable. Thus, behavioral and evoked potential correlates of easy and difficult discriminations could be studied, and the interactions with different times of day and other variables examined. EEG was recorded from vertex, frontal, temporal, and parietal sites by means of stainless steel screws implanted into the skull.

Initially, the intended terminal frequencies for all three tones were introduced and the monkey proved completely unable to learn the discrimination between the low and middle tone. Then the middle tone's frequency was elevated considerably until the discrimination was formed, and then gradually lowered again until it once more reached the terminal frequency; with this procedure the monkey displayed a high but not perfect amount of discrimination, permitting separate examination of evoked potentials for correct vs. incorrect responses. Analysis of the evoked potential data is not complete; however, the signals are clean enough to indicate that the recording methodology employed is feasible, and a full study to investigate this paradigm will be carried out when methods for on-line sorting and averaging of the data are available.

Operant blood pressure and avoidance conditioning in the baboon: A pilot protocol was approved for a study comparing operant diastolic blood pressure conditioning and Sidman avoidance as methods for inducing and studying chronic arousal in the baboon. As of this writing, the urine-collecting procedure for neuroendocrine data has been worked out; one of the animals is currently being habituated to the chair restraint, with a second being maintained in a free cage for initial neuroendocrine sampling. The computer programs to run the 12-hour daily sessions and record physiological and behavioral data are complete except for final debugging, and the exact procedures for the Sidman avoidance task are currently being worked out. The original protocol provided for shock as the negative reinforcement for avoidance. Although not yet certain, preliminary work indicates that it may be possible to use a loud noise generated by a Sonalert as the reinforcer, obviating the need to use shock. Currently a pilot baboon has learned to press a lever to escape the noise, and is currently being shaped onto avoidance performance. If reliable avoidance responding can be maintained, all future subjects will also have the Sonalert as a negative reinforcer, eliminating the ethical and practical problems associated with electric shock.

#### BASIC STUDIES OF STRESS RELATED AUTONOMIC FUNCTION

Pressor, cardioacceleratory and inotropic responses following electrical stimulation of the cat spinal cord: Changes in blood pressure, heart rate and cardiac contractility were produced in the cat following

electrical stimulation of the zona intermedia of the spinal cord. These cardiovascular responses were found to be well localized, and sites of maximal response were demonstrated histologically to lie within the intermediolateral nucleus (ILN). Pressor, chronotropic and inotropic responses were elicited from all spinal segments between T<sub>1</sub> and L<sub>4</sub>, i.e., the longitudinal extent of the ILN. Chronotropic and inotropic responses were markedly lateralized to the right ILN over the entire extent of the thoracolumbar preganglionic sympathetic column, whereas right sided pressor responses exceeded left sided responses only over the upper thoracic segments.

These findings confirm the right sided lateralization for sympathetic preganglionic cardioacceleratory neurons previously described but extend the origin of such neurons to the entire intermediolateral nuclear column. The right sided lateralization of the inotropic response and the differing pressor responses noted between upper thoracic and lower thoracic-lumbar segments have not been previously described.

An intraspinal sympathetic preganglionic pathway: physiologic evidence in the cat: Chronotropic and inotropic stimulation of T<sub>12</sub>-L<sub>2</sub> intermediolateral nuclear cells in the spinal cord of the cats which had undergone bilateral cervical vagotomy and C<sub>7</sub> spinal cord transection. The cardiovascular responses were unaffected by bilateral adrenalectomy or bilateral T<sub>11</sub> sympathetic chain transection, but were abolished by a second spinal cord transection at T<sub>11</sub>. These findings provide physiologic support for the existence of an intraspinal sympathetic pre-ganglionic pathway and suggest that this pathway may have a role in the control of heart rate and cardiac contractility.

Project 3E762771A804 MILITARY PSYCHIATRY

Work Unit 041 Behavioral variables in autonomic function and disease  
in military personnel

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5. Koob, G. Incentive shifts in intracranial self-stimulation produced by different series of stimulus intensity presentations. Physiol. and Behav., 18:131-135, 1977.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>2</sup>	2. DATE OF SUMMARY <sup>3</sup>	REPORT CONTROL SYMBOL	
				DAOC 6453	77 10 01	DD-DR&E(AR)636	
3. DATE PREV SURRY	4. KIND OF SUMMARY	5. SUMMARY SECY <sup>4</sup>	6. WORK SECURITY <sup>5</sup>	7. REGRADING <sup>6</sup>	8. DISEIN INSTR'N	9. SPECIFIC DATA-CONTRACTOR ACCESS	10. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES	<input type="checkbox"/> NO
10. NO /CODES <sup>7</sup>		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
a. PRIMARY		62771A		3E762771A804		00	
b. CONTRIBUTING							
c. CONTRIBUTING		Cards 114F					
11. TITLE (Proceed with Security Classification Code) <sup>8</sup>							
(U) Military Preventive Psychiatry							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>9</sup>							
003500 Clinical Medicine 013400 Psychology 021900 Physiology							
13. START DATE	14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY	16. PERFORMANCE METHOD			
76 07	CONT		DA	C. In-House			
17. CONTRACT/GRAANT		EXPIRATION:		18. RESOURCES ESTIMATE			
a. DATES/EFFECTIVE: N/A				FISCAL	B. PROFESSIONAL MAN YRS	C. FUNDS (In thousands)	
b. NUMBER:				PRECEDING	77	202	
c. TYPE:				YEAR	CURRENT	416	
d. AMOUNT:				78	6.5		
e. KIND OF AWARD:		f. CUM. AMT.					
19. RESPONSIBLE DOD ORGANIZATION							
NAME: Walter Reed Army Institute of Research Washington, DC 20012							
ADDRESS: <sup>10</sup>							
RESPONSIBLE INDIVIDUAL							
NAME: Rapmund, COL G. TELEPHONE: (202) 576-3551							
21. GENERAL USE							
Foreign Intelligence Not Considered							
22. KEYWORDS (Proceed EACH with Security Classification Code) (U) Psychiatric Illness; (U) Military Adjustment; (U) Environmental Factors; (U) Social and Psychological Factors; (U) Stress							
23. TECHNICAL OBJECTIVE <sup>11</sup> , 24. APPROACH, 25. PROGRESS (Punctuate individual paragraphs identified by number. Proceed text of each with Security Classification Code.)							
23. (U) This unit examines the dynamics of those specific factors within military organizations and environments that conduce to psychiatric illness, operate to produce psychiatric casualties and lead to the generation of dysfunctional behaviors and decrements in military performance. These studies have direct relevance for the development of programs of intervention and prevention and the development of effective techniques for the minimization of psychiatric casualties.							
24. (U) The methods of clinical psychiatry, social and clinical psychology, social anthropology and field epidemiology are used to identify factors that generate psychiatric casualties, behavior dysfunction and performance dysfunction and decrement in order to modify such factors or the relationships between them.							
25. (U) 76 10-77 09 In the course of this fiscal year data gathering in collaborative studies with USARIEM of Artillery Fire Direction Centers are being analyzed to determine behavioral factors that mitigate or contribute to the stresses generated by continuous performance. Further field and laboratory studies of social, psychological and physiological factors generated by the demands of sustained operations are in the process of development and should be initiated in this fiscal year. These studies will be carried out in collaboration with the Department of Military Medical Psychophysiology. A study of the relationship of social supports to chronic stress using dialysis patients was completed during this fiscal year. Studies of women in the Army and the social, environmental, and psychological factors effecting both the mental and physical health have been developed and field studies will begin during the course of this fiscal year. In these studies with the use of interview, questionnaire and observation, attempts will be made to delineate factors of risk for female personnel. These studies are projected in a 3 year prospective time frame. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 76 - 30 Sep 77.							
<small>*Available to contractors upon originator's approval.</small>							

DD FORM 1498  
1 MAR 68

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AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3E762771A804

Work Unit 042 Military Preventive Psychiatry

**Investigators.**

Principal: David H. Marlowe, Ph.D.

Associate: CPT George D. Bishop, MSC, MAJ Robert E. Blaik, MC,  
MAJ T. Peter Bridge, MC, E5 Joseph Bruer, William  
E. Datel, Ph.D., Rosemary A. Diliberto, MSW, SFC  
Jeremiah R. Dixon, Robert N. Dornhart, MA, LTC  
Juan M. Garcia, MC, MAJ Steven D. Gilbert, MC, CPT  
Eugene E. Grossman, MSC, Glenn T. Gurley, BA, LTC  
Jesse J. Harris, MSC; SSG Nelson Nelson S. Henry,  
MAJ William Hollinshead, MC; Richard Howard, MA,  
MAJ Larry H. Ingraham, MSC, E5 Patricia Kling,  
E5 Marie A. McCarty, MAJ David A. McFarling, MC,  
Richard J. Oldakowski, MAJ David W. Pearson, MC,  
Joseph M. Rothberg, Ph.D., CPT Robert J. Schneider,  
MSC, E5 JoAnne Smith, LTC Albert Tamoush, MC, SSG  
Charles I. Taylor

Description

Neuropsychiatric casualties have represented a major source of manpower loss in every armed conflict in which the United States Army has been involved. In times of peace the Army suffers significant personnel losses and costs as a function of behavioral dysfunctions, performance decrements, effectiveness deficits, psychosomatic illnesses, psychogenically based disorders and neuro-psychiatric diseases. Many of these losses and costs appear to involve predisposing risk factors that are parts of the general and human ecology of the Army. Unique aspects and demands of military life engender both strains and stresses that further the risk of the individual and the group for dysfunctional and ineffective behavior. The symptomatic and often costly responses to stressful events and factors in the military are in part determined by the health status and coping styles of the individual and in part by the social milieu in which stressful events are experienced. The interaction of the individual and group within this special set of ecological settings; ranging from the intense, life-threatening multiple stresses of combat to the daily stresses and strains of garrison and training represent the central concern of this work unit. This unit examines the dynamics of those specific factors within military organizations and environments that conduce to psychiatric illness, operate to

produce psychiatric casualties, and lead to ineffectiveness, the generation of dysfunctional behaviors, and decrements in military performance. Central to this concern is the study of how the Military social milieu organizes, shapes, reinforces and mitigates responses to stressful events. These studies have direct relevance for the development of programs of intervention and prevention and the development of effective techniques for the minimization of psychiatric and behavioral casualties.

1. Studies of Artillery Fire Direction Centers in Simulated Sustained Operations

Description

These studies carried out jointly with the Department of Military Medical Psychophysiology are dealt with in joint annual report under separate cover. These studies represented the major effort of this work unit during the past fiscal year.

2. Disordered Behavior and the Ongoing Family Milieu

Description

The goal of this study was the differentiation of military families in relation to (1) selected dimensions of intra-familial interaction (2) social network relationships outside of the family and (3) the classification of the presenting problems within a behaviorally descriptive framework. Structured and semi-structured interviews with seventeen military families for whom the "disordered" behavior of an adolescent precipitated a referral to the Child and Adolescent Clinic, Walter Reed Army Medical Center.

Progress

During the period of this report the data has been analyzed and a draft of the completed report has been drawn up. Thirty per cent of the present problems, which related generally, to dissension and/or antagonistic parent-child relationships, were attributed to family members other than the identified patient. Families were unable to mutually identify the exact nature of the primary problems for which child guidance help was being sought. However, they believed that changes, geared toward resolving such problems would have to involve changes made by the entire family. There was a clustering and/or differentiation of the families in relation to the following: (1) the number of reported presenting problems, (2) the extent to which family members were able to mutually identify "the problem(s) for which child

guidance help was sought, (3) the risk to the family for the development of psychiatric symptomatology related to the occurrence of stressful recent events, (4) the amount of perceived isolation in the home and (5) the magnitude or extreme nature of past and/or current problems affecting the family unit. Perhaps, the most interesting finding that extends across 16 or 94% of the families was their recurring history of family problems. The nature of such problems had, in most instances, influenced referrals to child guidance or mental hygiene clinics, social workers, psychologists, psychiatrists, alcoholics anonymous, etc. Data strongly indicate that there was variable amounts of "problem-turbulence" smoldering within these families. The disordered family system remains a reality, however, the role of identified patient has shifted from one family member to another. Research questions which relate to this phenomenon are: What factors relate to the problem-proneness of these families. What factors relate to the overt manifestation of problem situations which are pervasively covert within these families. What factors contribute to the choice and/or labeling of an identified patient within these families. Families differed from each other relative to the amount of time family members believed that they were alone. However, more "perceived isolation" or separateness occurred in families having a female identified patient than it occurred in families having a male identified patient. A question raised by the data is whether there are operational dynamics related to separateness, estrangement, seclusiveness, alienation, isolation, lonesomeness, etc. which exist more prominently in families of female identified patients than in families having a male identified patient.

In general, many of the families spent a great deal of their time apart from each other, even when they were all present in the home. In families in which "territoriality" was in operation, "personal" television sets and varied activities in separate bedrooms served as barriers to intra-familial interaction. Accordingly, "dens", "family rooms", "the basement", "garage", etc. were places sequestered by family members of the family unit.

There were several serious limitations involved in this study. Perhaps the first and most crucial was the lack of control families, with which the "problem families" could be compared. Second, the number of variables considered for measurement was too extensive and third, as each section of the study ran parallel to other sections of the study, it was extremely difficult to measure aspects of interaction between the variables to each other across families.

### 3. The Psycho-Social Aspects of Health and Illness of Women in the Army

#### Description

The purpose of this study, which is presently under development, is to describe the relationship between various psycho-social and organizational variables and the health status and effectiveness of performance and functioning of women in the Army. As of 1979, the Army plans to have approximately 50,400 enlisted women in its ranks many serving in non-traditional MOS's. The impact of such increases in the number of female personnel on health resources, the possible consequences for individual and unit mental health and integrity are, at present difficult to ascertain. Recent data on psychiatric hospitalization rates shows that for 1971-73 the rate of episodes of psychiatric hospitalization for women was approximately twice that of males if drug and alcohol categories are excluded. Provisional analyses have shown that Army women had more days lost than men and a greater non-effective rate in 1975 counting both physical and psychiatric hospitalizations. When gender specific illnesses were excluded, females continued to have a higher hospitalization rate than males. Similar results have been shown for the Navy. Past experience indicates that when mental health rate differentials exist for groups in the Army, major contributory factors are invariably to be found in organizational and unit factors and in psycho-social factors - particularly the stress provoking ones found in the military environment. The study under development projects extensive work in the field to: determine if such factors exist; isolate and describe them;; and consider preventive measures.

#### Progress

This study is presently in its planning phase. Initial bibliographic work has been completed and will be followed by a review paper covering what is already known of psycho-social and environmental factors affecting women in the military. Initial drafts of the final research protocol are nearing completion, and interest and support have been extended by the WAC Directorate. It is anticipated that the field phase of this study will begin during the first half of FY 78.

### 4. The Relationship between Social Support System and Chronic and Acute Stress

#### Description

Contact was made with a panel of 19 Hemodialysis patients

and their families. These individuals were interviewed and followed to determine the relationship between coping styles, social support system response and the ability to cope with life threatening stress (i.e., renal failure). It was theorized that the hemodialysis patient might provide an analogue for the study of response and response mediation to the kinds of life threatening stresses seen on the battlefield.

Progress

The data collection phase of this study has been completed. Data analysis is presently being done by the senior investigator who has left the service. It is anticipated that a final report will be available in the first half of FY 78.

PUBLICATIONS

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- Marlowe, D.H., "Review: migrants of the mountains by W.R. Geddes." Journal of Asian Studies, Vol XXXVI, No 3, 1977, pp. 591-2.
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- Schneider, R.J., Kojak, G. and Resdorf, J. "Father distance and drug abuse in young men." J Nervous Mental Disorders, Vol 165, No 4, 1977.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>8</sup> DA OC 6456	2. DATE OF SUMMARY <sup>8</sup> 77 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMRY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY <sup>9</sup> U	6. WORK SECURITY <sup>9</sup> U	7. REGRADING <sup>9</sup> NA	8. DRG'D INSTR'N NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO. / CODES: <sup>10</sup> a. PRIMARY 62771A	PROGRAM ELEMENT PROJECT NUMBER 3E762771A804			11. LEVEL OF SUM WORK UNIT NUMBER 00 043		
b. CONTRIBUTING						
c. CONTRIBUTING	CARDS 114F					
11. TITLE (precede with Security Classification Code) <b>(U) Military Stress: Health, Performance and Injury Factors</b>						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>11</sup> 016200 Stress Physiology 013400 Psychology						
13. START DATE 76 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House			
17. CONTRACT/GRANT NA	18. RESOURCES ESTIMATE EXPIRATION:	19. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research ADDRESS: Washington, DC 20012	20. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Division of Neuropsychiatry ADDRESS: Washington, DC 20012	21. GENERAL USE Foreign intelligence not considered	22. KEYWORDS (precede EACH with Security Classification Code) (U) Electrophysiology; (U) Psychophysiology; (U) Psychophysics; (U) Stress; (U) Performance; (U) Human Volunteer	23. TECHNICAL OBJECTIVE, <sup>12</sup> 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23. (U) Stressful environments, performance demands and physiological conditions likely to produce significant deterioration in the health and performance capacity of soldiers are studied. The psychological and physiological functions that contribute to decrements in health and performance are identified and therapeutic and prophylactic strategies are developed. Special attention is paid to situations involving rapid deployment and continuous performance. 24. (U) Psychophysiological and behavioral methodologies are used to isolate, analyze, and control endogenous and exogenous factors contributing to health, disease and performance capacity. 25. (U) 76 10 - 77 09 In vivo and in vitro studies of the mode of operation of an in-house developed infra-red photoplethysmograph were accomplished. Results indicate that the device responds to changes in the optical orientation of blood cells and is linearly related to blood flow over a physiologically useful range. Other work dealing with the non-invasive monitoring of cardiac function, chronic pain related to peripheral nerve injury, and the relationship between information processing and the cardiac cycle was accomplished. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 76 - 30 SEP 77.

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\*Available to contractors upon originator's approval.

DD FORM 1498  
1 MAR 68

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AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3E762771A804 MILITARY PSYCHIATRY

Work Unit 043 Military Stress: Health, performance and injury factors

Investigators.

Principal: Frederick W. Hegge, Ph.D.  
Associate: LTC Albert J. Tahmoush, MC; CPT John R. Jennings,  
MSC; John Schrot, Ph.D., Alison L. Lee; Stanley  
Hall, B.A.; George Lampron; Helen Sing, M.S.

Description

The soldier must perform in a multi-stress environment which affects health and performance. Research under this work unit is directed to a description of the stress factors, determination of the effects of these stressors on health and performance, and the design and implementation of counteractive measures to minimize these effects. Special attention is paid to stresses found in combat such as continuous performance, sleep deprivation, and temporal disorientation. The incidence of neuropsychiatric injuries and disease in a military population, the characteristics of these illnesses, counteractive measures to improve recovery, and the effect of residual deficits on performance are addressed in this unit. Performance is viewed as a continuum of human activity ranging from simple motor behavior to the most complex activity. A major attempt is made to distinguish between variations in performance which reflect: a) changes in an individual's basic sensitivity, efficiency, or capacity to perform a given task; and b) changes in the response biases, response, criteria, or strategies employed in task performance. The research strategy utilized field studies for the delineation of stress factors and their effects on health and performance, plus laboratory studies to test experimental analogs and to develop counteractive measures.

Progress

1. Clinical Studies on Causalgia

In the 1976 Annual Report, a historical review of the reported literature on causalgia was performed. The absence of necessary and sufficient criteria for the diagnosis of causalgia has led to considerable confusion as well as diversity in the cases reported. Review of the literature suggested that the minimum criteria required for diagnosis are: (a) the occurrence of a traumatic event hours or days prior to the onset of pain; (b) the presence of continuous, burning pain distal to the site of trauma; and (c) the presence of hyperalgesia and dysesthesia in the cutaneous distribution of pain. The clinical course and associated findings in fifteen patients who met these criteria were detailed in the 1976 Annual Report.

Eight of the fifteen patients with a diagnosis of causalgia completed the McGill pain questionnaire (22,23). This questionnaire contains three classes of word descriptors -- sensory, affective, and evaluative -- that may be used to specify subjective pain experience. Completion of the questionnaire provides three measures: (a) the pain rating index (PRI) based on the numerical values assigned to each word descriptor; (b) the number of words chosen (NWC); and (c) the present pain intensity (PPI). For the patients with causalgia, the mean sensory PRI was 22.6; the mean affective PRI was 3.44; and the total PRI was 39.4. The mean NWC was 14.5, and the mean PPI was 4.2. As seen in Table 1, the means for causalgia were higher for each measure than those reported for patients with post-herpetic pain, arthritis, and dental pain (23).

**Table 1.** Mean pain rating index (PRI), number of words chosen (NWC), and present pain intensity (PPI) for patients with causalgia, post-herpetic pain, arthritis, and dental pain.

<u>Pain syndrome</u>	<u>N</u>	<u>Mean PRI</u>	<u>Mean NWC</u>	<u>Mean PPI</u>
causalgia	8	39.4	14.9	4.2
post-herpetic pain	5	22.6	10.4	3.0
arthritis	19	18.8	8.1	1.9
dental pain	15	19.5	8.3	2.3

In order to compare the clinical findings of patients with causalgia in a non-combat population to those in a combat population, the medical records of soldiers injured during the Vietnam conflict who had this diagnosis are being examined. Unfortunately, the majority of these records do not contain sufficient information to substantiate the diagnosis according to the necessary and sufficient criteria listed above. Fifteen records did contain the information required, and work is in progress to compare the clinical findings in the two groups.

## 2. The Photometric Examination of Vascular Events

Optical techniques for the examination of vascular events have been classically described as photoplethysmography, and previous work in this area was reported under that terminology. However, this technique is basically a measurement of light intensity (photometry). The monitoring of vascular events is accomplished through the changes in backscattered light intensity produced by vascular activity. The optical foundations of this technique have been presented in a chapter to be published in the book "A Manual of Psychophysiological Methods" by Venables and Martin.

The photometric technique provides an index of blood flow, and not changes in the size of blood vessels as the term photoplethysmography implies (Pub. 4). The theoretical foundations relating changes in blood flow to changes in the amount of backscattered light were presented in the 1976 Annual Report. Experimental confirmation of the theoretical formulations have been obtained during the last year.

With steady flow of blood through a non-compliant glass system, there is an increase in the backscattered light signal for increases in steady flow. With steady flow of plasma or hemolyzed blood, there is no change in the backscattered light intensity for changes in steady flow.

These in vitro suggest that the assumptions of photoplethysmography are invalid. If the optical signal is solely determined by volumetric changes in the vessels under examination, one would not obtain an optical signal from a system which is constrained to be volumetrically constant. As discussed in the theoretical foundations presented in the 1976 Annual Report, orientation of intact red blood cells is the most likely mechanism by which changes in backscattered light intensity are produced by changes in blood flow.

### 3. Causalgia. Measurements of Sympathetic Nervous System Activity in Patients and Matched Controls

Background material and preliminary results concerning the measurement of skin temperature, skin conductance, and an optical index of blood flow in the affected and contralateral non-affected extremity of patients with causalgia and of controls were presented in the 1976 Annual Report. Ten patients and ten controls have been studied, with follow-up data on six patients after significant relief of pain.

A summary of the clinical characteristics of the ten patients is presented in Table 2. Patient selection was based on fulfillment of the necessary and sufficient criteria for diagnosis discussed in the section on clinical studies. The patient group was subdivided according to previous treatment: (a) patients 1 - 4 had no prior sympathetic nervous system intervention; (b) patients 5 - 7 had sympathetic blocks; and (c) patients 8 - 10 each had a unilateral sympathectomy.

The measurements of skin temperature, skin conductance, and skin blood flow index were performed on the affected and non-affected extremity on two consecutive days during two thirty-minute sessions on each day. The transducers were switched between sessions on each day. The mean and standard error for each measure is presented in Fig. 1 for the controls, and in Fig. 2 for the patients.

As shown in the 1976 Annual Report, the skin temperature measure generally varies over a very small range (less than one degree Fahrenheit) during a session. The skin conductance and skin blood flow index may vary over a wide range during a session. In order to determine if significant limb differences were present in patients and controls, a within subject factorial analysis of variance was performed for limb effects, day effects and transducer effects. In the control group, significant limb differences between affected and non-affected extremities were noted only for skin temperature in control 6 ( $p < .001$ ). Significant day differences were noted for skin temperature in Control 2, 7 and 8 ( $p < .001$ ), Control 9 ( $p < .05$ ), and Control 10 ( $p < .01$ ).

Table 2. Clinical Characteristics of Causalgia Patients

<u>PATIENTS</u>	<u>AGE, SEX</u>	<u>PRECIPITATING FACTOR</u>	<u>DURATION OF PAIN</u>	<u>PREVIOUS TREATMENT</u>	<u>AUTONOMIC ABNORMALITIES IN AFFECTED EXTREMITY</u>
Case 1 (P)	49, F	Transverse Carpal Ligament Release	18 Months	Analgesics, P.T.	None
Case 2 (G)	45, F	Fasciotomy of Hand	4 Months	Narcotics, P.T.	Cold, Plethoric, Trophic changes
Case 3 (R)	51, F	Lumbar Laminectomy	12 Months	Narcotics, P.T., Nerve Exploration,	None
Case 4 (M)	52, F	Neck Injury	1 Month	P.T.	Sweaty, Plethoric
Case 5	40, F	Multiple I&D	10 Months	Sympathetic Blocks	None
Case 6 (We)	25, M	Shrapnel Injury	7 Years	Analgesics, Nerve Exploration, Sympathetic Blocks	Sweaty
Case 7 (Y)	35, M	Compound Fracture of Foot	4 Years	Sympathetic Blocks	Warm, Dry, Plethoric
Case 8 (F)	34, M	Gunshot Wound	7 Years	Stellate Ganglionectomy	Trophic changes
Case 9 (Wa)	28, M	Ankle Injuries	2 Years	Stellate Ganglionectomy	Dry, Warm
Case 10 (C)	47, M	Gunshot Wound	3 Years	Stellate Ganglionectomy Thalamotomy	Cold, Dry, Constricted, Trophic changes

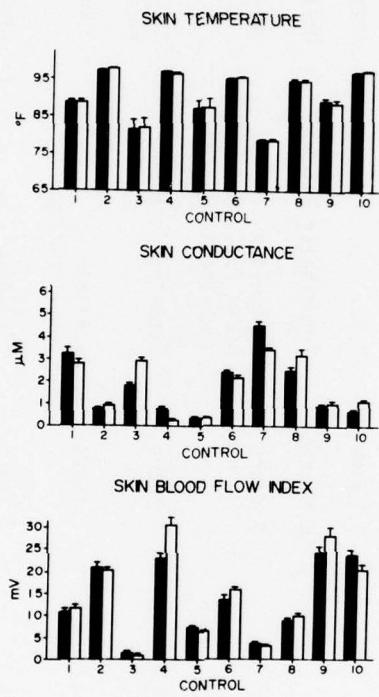


Figure 1. Mean + S. E. for measures of skin temperature, skin conductance, and skin blood flow index performed in ten controls.  
 affected extremity.      non-affected extremity.

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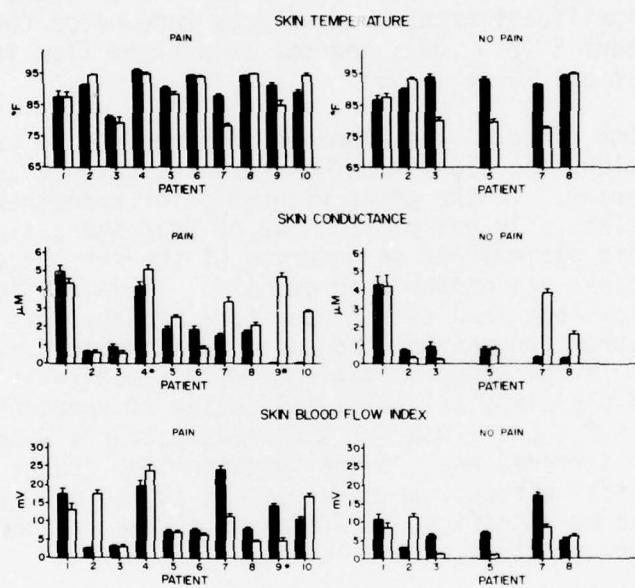


Figure 2. Mean + S. E. for measures of skin temperature, skin conductance, and skin blood flow index performed in ten patients with causalgia and in six of these patients after relief from pain. affected extremity. non-affected extremity.

Significant day differences were present for skin conductance in Control 1 ( $p < .05$ ), and for skin blood flow in Controls 2, 3, 7 and 8 ( $p < .001$ ). Significant transducer effects were noted only for skin temperature in control 6 ( $p < .001$ ).

In the patient group when pain was present, significant limb differences were present for skin temperature in Patients 2, 7, and 10 ( $p < .001$ ), and Patient 8 ( $p < .05$ ). For skin conductance, significant limb effects were present in Patients 7 and 9 ( $p < .01$ ), and Patient 10 ( $p < .001$ ). For skin blood flow, significant limb differences were present in Patient 2 ( $p < .001$ ), Patient 7 ( $p < .01$ ), and Patients 9 and 10 ( $p < .05$ ). Significant day effects for skin temperature were noted in Patient 8 ( $p < .01$ ) and Patient 9 ( $p < .05$ ). For skin conductance, significant day effects were noted in Patients 4 and 8 ( $p < .01$ ). Significant transducer effects were noted for skin conductance in Patient 5 ( $p < .05$ ), and for skin blood flow in Patient 5 ( $p < .01$ ) and Patient 10 ( $p < .05$ ).

When the limb effects were examined with respect to prior sympathetic intervention, the significant differences noted cluster according to prior therapy. In the group without prior sympathetic nervous system intervention, only one patient out of four had a significant limb effect. This patient had an aneurysm of the subclavian artery with surgical repair and probable thrombosis. The significant difference in blood flow index and skin temperature probably was due to large vessel obstruction and not increased sympathetic nervous system (SNS) activity. In the group of patients with sympathetic blocks, Patient 7 showed the classical triad indicative of sympathetic denervation (+ S.T., + S.C., and + S.B.F.I.) whereas patients 5 and 6 showed no significant limb differences. In the sympathectomy group, patient 9 showed the classical effects, whereas patient 10 had the typical S.C. + and patient 8 had no significant differences in the measures probably due to inadequate sympathetic denervation.

Six patients had repeat measures performed after relief of pain. Patients 1 and 2 received propranolol with pain relief but no change in the physiological measures. Patients 3, 5, 7, and 8 had a unilateral stellate ganglionectomy with pain relief. Evidence of sympathetic denervation was present in each of these patients, although the classical triad was again noted only in patient 7.

Although the patient sample is small, this study suggests that sympathetic nervous system mediated variables, such as skin temperature, skin conductance and skin blood flow, do not play a pathophysiologic role in this pain syndrome. Causalgia may be present with varying combinations of SNS activity, and relief of pain is not dependent on a specific change in the levels of these SNS mediated variables.

#### 4. Battle Injuries and Associated Neurologic Disorders Sustained by Active Duty Army Personnel During the Vietnam Conflict

The combat effectiveness of a military population, in part, depends

on an understanding of the medical effects of combat and the development of counteractive measures to minimize these factors. A descriptive analysis of battle injuries sustained in previous conflicts may be of value in predicting the effects of combat on health. A cooperative research proposal with the Department of Military Psychiatry approved to perform an epidemiologic study of battle injuries and associated neurologic disorders sustained by active duty Army personnel during the Vietnam conflict. Work has begun on the formation of a medical data file of wounded Army personnel.

## 5. Speed-accuracy Tradeoff: Performance and Physiology

### A. Development of Speed-accuracy Tradeoff Functions as Performance Measures.

Response latency and accuracy are basic aspects of virtually all human performance. Latencies are frequently found sensitive to changes in performance requirements and to environmental stressors. As such, latencies or reaction times have become a central measure for the assessment of performance. This is particularly true of military task performances where accurate, rapid response may be of critical importance.

Accurate, rapid responding is not, however, a simple concept. Although instantaneous, completely accurate responding may be desirable; this is impossible even in the simple situation of lifting one of two fingers depending on whether a high or low frequency tone occurred. Individuals may be able to produce essentially instantaneous responses (less than 100 msec); but these responses will not accurately reflect which tone occurred; on the other hand, relatively slow responding (greater than 400 msec) will allow essentially perfect accuracy. The individual must decide on a strategy or tradeoff which yields a speed and accuracy which he judges appropriate to the functional requirements of the task.

We define the basic capability of the individual to sense external events, produce responses, and coordinate these functions as his performance efficiency. The strategy or tradeoff judgement which he makes, we refer to as his response criterion. Efficiency and response criterion can be independently influenced by task conditions. For example, the time required for an individual to detect the occurrence of a target might be increased under stress for either of the following reasons: a) because stress decreases basic performance efficiency; or b) because a more conservative criterion for responding has been adopted under the stressful conditions. These two alternative explanations would require very different means for counteracting the stress-induced decrement in performance, yet conventional experimental procedures are powerless to distinguish between them. Experimental procedures such as a speed-accuracy tradeoff paradigm or related procedures derived from the theory of signal detection must be employed (6). Using a signal detection approach Broadben, for example, (2) has suggested

that changes in vigilance performance under numerous conditions will be due to changes in criterion rather than detection efficiency.

We have contributed to the development of speed-accuracy tradeoff functions as performance measures which allow the separate assessment of performance measures which allow the separate assessment of performance efficiency and response criterion. By inducing individuals to systematically vary their relative emphasis on speed versus accuracy (i.e., their speed-accuracy criterion), a speed-accuracy tradeoff function may be derived which expresses the functional relationship between RT and accuracy over a range of criteria (29,34). Such functions may be thought of as operating-characteristics for response latency in the same sense that the functional relationship between hit rate and false alarm rate constitutes an operating-characteristic for signal detection performance (18, 31). Changes in an individual's speed-accuracy criterion are represented by shifts in performance along a single speed-accuracy function. In contrast, changes in performance efficiency are represented by performance shifting from one speed-accuracy tradeoff function to another. We (11) have previously found, for example, that acute alcohol intoxication has a clear effect on performance efficiency.

Experimental and computational differences between different speed-accuracy tradeoff paradigms have important interpretive implications. We have compared two paradigms those for the speed-accuracy tradeoff function (SATF) and the conditional accuracy function (CAF) (34). A SATF is created by collecting data under different speed emphasis conditions and then basing the computation of the tradeoff function on mean accuracy and reaction time for those conditions. A CAF is created by allowing individuals to freely vary speed and accuracy and then classifying the data into categories based on obtained reaction time (34). If one particular model, the adjustable timing model, holds; Ollman (28) has shown theoretically that the CAF and the SATF are identical and that the CAF is the more general function. Intuitively this model suggests that the accuracy of the response is solely determined by the timing of the response. This model implies that CAF's from different speed emphasis conditions should have similar parameters and should be similar to a CAF or SATF computed across those speed-emphasis conditions.

This implication was questioned in the above report (34) and continues to be questioned in currently collected data. Our approach suggests that the speed emphasis condition determines an average criterion for amount of information available prior to response initiation. Variability in the maintenance of this criterion is a possible source of variation in speed and accuracy within a speed emphasis condition. A second potential source of variation is variation in efficiency -- from trial to trial information is slow to accumulate or response preparation is not achieved on time. Previous work (34), suggested that variability within speed emphasis conditions and the relative speed of correct and error responses were the primary sources of differences between the CAF and the SATF. For this reason, it is

empirically and theoretically important to understand the relative role of efficiency and criterion variability in speed-accuracy paradigms.

Current work manipulates the variability of criterion and efficiency in a choice reaction time task to observe the relative influences of these factors. Criterion is varied between payoffs for a narrow range of reaction time (+ 25 msec) and a wide range (+ 50 msec). Efficiency is varied indirectly by allowing tone loudness to vary independently of frequency, the discrimination controlling the choice reaction. Lappin & Disch (19) have previously shown that light intensity influences efficiency rather than speed-accuracy criterion. Results to date support the efficacy of loudness in varying efficiency, but data is not available for assessment of the primary questions posed by the experiment.

#### B. Cardiac Cycle Effects: Vagal-time Interacts With Response Timing

Both neurophysiological and performance studies have reported a phasic variation in the organism's arousal or capability to perform that is synchronized with the cardiac cycle (1,16). The Laceys' (7) for example, have reported that second to second increases in inter-beat interval are associated with relatively rapid reaction times and that the magnitude of this correlation is a function of the timing of the reaction time stimulus relative to the R wave of the electrocardiogram. These results suggested that the cardiac cycle-arousal-reaction time relation might be a microcosm of more general relations between physiological function, neurophysiological arousal and performance.

A recently published experiment (publication 1) investigated the relation of cardiac cycle time of stimulus presentation to efficiency and criterion measures derived from the SATF. The results of this work were described fully in last year's annual report. Briefly, the cardiac cycle time of the completion of a reaction time was found to exert a clear influence on the cardiac inter beat interval; but performance indices were not dependent on cardiac cycle time. Previous work (27) had indicated the primary role of the vagus in control on inter-beat-interval in this type of task. Thus, our results indicated a direct interaction between timing of the vagal control of inter beat interval and a psychological event -- completion of a choice response.

The timing of vagal inhibitory input to the pacemaker of the heart varies with time in the cardiac cycle (4,12,14,21). Figure 3 presents this diagrammatically: an initial inhibitory peak occurs between 200 and 300 msec after the R wave; a second peak may occur late in the cycle (beyond 700 msec from R wave). The experiment just discussed only sampled a few time points within the cardiac cycle. Further work was required to parametrically explore time within the cardiac cycle. The experiment was further directed at separating

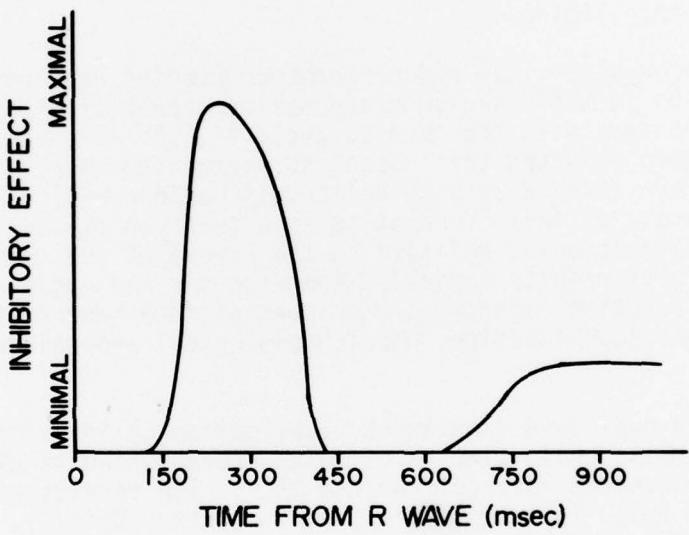


Figure 3. Diagrammatic representation of the timing of vagal input to the human heart. The representation is an extrapolation from animal studies.

stimulus and response influences on the obtained cardiac cycle effect. Eight cardiac cycle times between 0 and 525 msec from R wave were investigated using a speed-accuracy tradeoff paradigm.

The results were in accordance with expectations derived from the time course of vagal timing. Figure 4 presents the results for a single subject. The ordinant of the figure, the inter beat interval for Beat 3 - Beat 4, may be viewed as an index of the degree of shortening of the IBI induced at the time of response completion. Stimulus timing was based on the R wave of Beat 4. The abscissa is time between the R wave and the completion of the choice response. Each point is the mean of 64 trials comprising one quintile of the trials from a cardiac cycle time condition. Quintiles are based on the speed of reaction time. Thus, one point, for example, is the second fastest quintile of the 75 msec-from-R wave cardiac cycle time condition. This point is plotted on the abscissa at 75 msec from the R wave plus the mean of the reactions times in the quintile, 231 msec, -- that is at 306 msec from R wave. A second degree polynomial has been fit to the data.

We interpret Figure 4 as showing the cessation of vagal firing induced by the completion of a choice response. Moving in time away from the R wave (from left to right in Figure 4) Beat 4 is initially shorter than Beat 3. The response to the choice stimulus has occurred prior to peak vagal inhibition and, according to our interpretation, prevented the occurrence of the inhibition. Beat 4 is not inhibited -- slowed -- and thus its inter beat interval is shorter than that of Beat 3. Moving to the 300 to 550 msec from R wave region of Figure 3, we see that Beat 3 is becoming progressively shorter than Beat 4. The response to the choice stimulus allows more and more of the vagal inhibition to occur as it moves in time through the peak period of vagal inhibition. Late in the cardiac cycle, beyond 700 msec, a recovery is suggested with Beat 3 not being quite as relatively short as previously. This suggests some decay over time of vagal inhibition.

Statistically significant and mathematically similar quadratic fits to the data were found in three of the five participants in the experiment. Additional analyses verified the relative importance of response timing over stimulus timing. No major influences of cardiac cycle time on performance were observed.

In sum, these experiments have developed a clear model of a potentially important and relatively direct influence of psychological events on neural control of the heart. From the perspective of our orientation toward performance, however, we have not found a performance task which is influenced by the cardiac cycle manipulation.

#### C. Efficiency and Criterion Effects in Performance Under Speed Stress

Last years' annual report suggested that an important source of

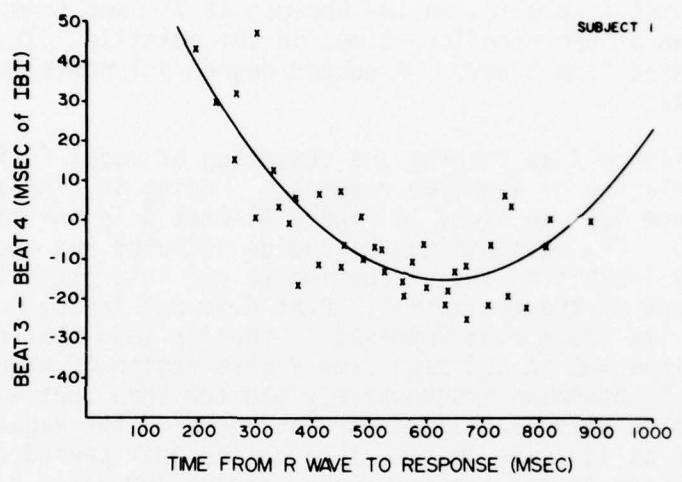


Figure 4. Change in inter-beat-interval during task performance as a function of cardiac cycle time.

error in military tasks involving time pressure might be an excessive reliance on expectancies to the exclusion of available stimulus information. This speculation was based on changes in criterion with high speed emphasis conditions in a speed-accuracy tradeoff task varying a priori probability of choice stimuli. Some support of this speculation was seen in a simulation of a Fire Direction Center under combat conditions conducted in conjunction with artillery units from the 82nd Airborne Division. In a continuous performance situation with the requirement to rapidly process firing information, these soldiers tended to frequently use digits from Fort Bragg map coordinants in their firing data rather than the actual data. Speed-accuracy tradeoffs at a more general level than the current discussion were also assessed in the Fire Direction Center study. Details of this are found this year in the work unit 125. Publication of the results for the experiment varying a priori probability has not yet occurred.

#### D. Efficiency and Criterion Effects in Performance Under Heightened Criticality of Task

A relatively mild, but effective stress induction is to increase the benefits and costs of performance. In the military this may vary from the stress induced by a peacetime commander who ties together personnel advancement and mission accomplishment to the vital potential benefits and costs of efficient combat performance.

In the speed accuracy tradeoff situation benefits and costs may be readily manipulated by payoffs used to maintain speed emphasis conditions. The criticality of accurate performance within the desired speed range may be increased by raising the positive reward and adding a penalty for responses which are inaccurate or out of the desired speed range. Note that the same behavior is rewarded as previously -- thus a change in average criterion should not be induced. Changes in efficiency might be expected; however, efficiency may improve if optimal performance has not been previously achieved or efficiency may decrease if the heightened criticality of the task induces an affective state which is not consonant with optimal performance (20,24).

The influence of task criticality on the individual performing the task may be assessed by self report as well as by an assessment of physiological reactions during the task. The choice reaction time task is associated with well known and very clear changes in the cardiovascular system (9,16,17). As noted above inter beat interval, in particular, becomes temporally linked to task performance. Obrist (25) has reported the basically vagal control of inter beat interval in this task, but has also shown evidence of sympathetic involvement when, in our terms, task criticality is increased. By assessing a cardiovascular measure primarily influenced by the sympathetic system, peripheral blood flow, and one primarily influenced by the parasympathetic system, inter beat interval, we hope to see a patterned cardiovascular response to heightened task criticality. Sympathetic

influences during heightened task criticality should be seen in peripheral vasoconstriction. In participants reacting strongly to the criticality manipulation, dissolution of the temporal synchrony between inter beat interval and performance may be observed.

In summary this experiment, which is currently in progress, should provide a fine grained assessment of the influence of a mild stress on ongoing performance and the normal autonomic nervous system involvement in that performance. The sensitivity of the SATF measures should allow a clear examination of the association or disassociation of autonomic and performance events under stress.

#### E. Efficiency and Criterion Effects in Speech Discrimination

Over the last three years we have investigated the role of discrimination efficiency and response bias in speech discrimination. The results, which are summarized in last years' annual report, have pinpointed aspects of speech discrimination that are differentially related to efficiency and criterion effects. This work has recently been published (Publication 3).

#### 6. Memory Load and Cardiovascular Responsivity: A Signal Detection Analysis

Studies of autonomic correlates of memory conducted in this laboratory have focussed on the relationship between changes in cardiac inter-beat-interval (IBI) and attentional requirements of various cognitive tasks or processes. These studies have suggested that: a) memory requirements produce relatively greater cognitive load than cognitive manipulation requirements; and b) such memory requirements also produce a consistent inhibition of cardiac deceleration and a maintenance of acceleration (8,10). These results may be set in two general contexts differing in emphasis but not necessarily mutually exclusive. First, the apparent cognitive load induced by memory requirements may be viewed as a stressor and the cardiac responses as an acute response to this stress. Second, the autonomic nervous system may be viewed as primarily concerned with regulation of the organism's effort. Cardiac acceleration is then interpreted as a reflection of the organism's adjustment to a momentary processing load, and will vary in magnitude with the magnitude of the load. The first view would suggest a correlation between cardiac IBI and performance errors and might predict changes in response bias as a result of the induced arousal. The second view suggests that cardiac IBI would be a function of processing load even under conditions of errorless performance and might predict a positive correlation between cardiac acceleration and memory performance.

In recent experiments (7), we directly examined the relation between cardiac IBI and memory using a recognition memory paradigm. Memory load was manipulated by varying set size between 5 and 10 simultaneously presented two digit numbers. After a five-second retention

interval, a single probe item was presented which might or might not be a member of the set presented. As well as judging whether or not the probe item was a member of the set, the subject rated confidence in that decision. Signal detection analysis of the recognition data revealed an expected monotonic decrease in detection with increasing memory load.

Variation in averaged sec. x sec. IBI showed reliable patterning during the memory task. Cardiac deceleration was a consistent correlate of the anticipation of important intratrial events (i.e., presentation or removal of memory set or recognition probe). Cardiac acceleration was associated with times corresponding to initial storage and subsequent rehearsal of memory items.

Neither the pattern nor magnitude of IBI changes varied with memory set size, nor were cardiac changes related to signal detection indices. Therefore, an interpretation of cardiac acceleration in terms of memory load was not supported by the data. However, subjects reported using strategies to limit memory load during the memory task (e.g., selecting for attention and rehearsal only a small subset of items from a large memory set). The probable consequence of such strategies, if frequently used, would be to weaken any effect of the memory load manipulation and to alter the magnitude (but not the observed pattern) of signal detection indices.

A follow-up study in progress uses the identical memory load manipulation but requires recall rather than recognition of memory items. This experiment is an attempt to determine whether the failure of memory load and memory performance to relate to cardiac acceleration was specific to the response requirements and performance measures of the recognition task. Currently available data are insufficient for analysis of this issue.

#### 7. Multiple Task Assessment of Processing Capacity

Performing two or more tasks at the same time is difficult and stressful for most people. Situational factors and the ability to allocate our attention determine whether all tasks show a decrement, whether performance on one is maintained at the expense of others, or whether some other strategy of attention allocation is employed. Man's limited ability to perform concurrent tasks suggests that his capacity to process information is limited and, perhaps, fixed (2,13,15). The psychological problem of attention may be posed in the context of such a limited capacity model. Given that only a limited amount of information can be processed, only a small portion of the available environmental and cognitive information must be selected for current processing. In order to understand the behavior of the organism, we must understand what aspects of the situation the organism has selected -- i.e. is attending to. In the context of a limited processing capacity model, we can further ask how much processing capacity a particular task employs, i.e., tapping a finger requires less capacity than guiding a missile.

As a working hypothesis it seems reasonable to suggest that tasks, which require a relatively large amount of processing capacity are more vulnerable to the influence of stress than tasks which require less capacity. At another level of abstraction we can talk of the processing requirements of cognitive processes integral to task performance. On the basis of this hypothesis it is important to measure the processing capacity requirements of different processes and observe the decrements in these processes when the required amount of capacity is unavailable.

In an experiment described briefly in last year's report and more fully here (10), a psychophysiological paradigm for the assessment of processing capacity allocation was developed and tested. Serial learning was the basic performance task. Allocation of capacity within this task was assessed with a concurrent probe RT task and with concurrent measures of cardiac inter-beat-interval. The rationale for the concurrent RT task is based on the limited processing capacity notion. Performance of a primary task, such as serial learning, requires a portion of the available processing capacity. The remaining capacity may be termed spare capacity available for performance of a second task. If a second task is introduced which requires somewhat more capacity than is available as spare capacity, performance on this secondary task will be relatively poor. Variations in processing capacity allocated to the primary task may then be measured by observing variations in the performance of the secondary task. The practical application of this paradigm requires that the secondary task be presented infrequently so it retains its secondary status and serves as a probe of the capacity allocated to the primary task (15).

The rationale for the measurement of inter beat interval is the sensitivity of this measure to requirements for the anticipation and careful observation of environmental events (3,8,9,17). Cardiac IBI has been shown in a variety of tasks to lengthen briefly during periods of attentive observation and shorten during the cognitive processing of information. The magnitude of these brief cardiac changes seems related to the involvement of the participant (16). Taken together these observations suggested the possibility that brief changes in cardiac inter beat interval might be related to processing capacity.

Probe RT and cardiac IBI were measured during serial learning over three trials employing multiple eleven item lists. The results confirmed the basic covariation of probe RT and IBI: probe RT increased and IBI decreased during actual learning as opposed to a control sequence and during the recall period as compared to the learning period. Furthermore, probe RT decreased and cardiac IBI increased over trials for items correctly recalled. Lengthening of IBI seemed to reflect the holding available of processing capacity for expected input; while the shortening of IBI was related to the use of processing capacity. Differences in probe RT and IBI for items correctly and incorrectly anticipated suggested that correct anticipation was associated with a larger allocation of processing capacity. Second to second changes in capacity allocation were related strongly to the mechanics of

task performance (response preparation and stimulus observation) rather than to the operation of any specific cognitive process.

The primary significance of these results is the demonstration of the basic convergence of probe RT and IBI as measures of processing capacity allocation. These measures suggested that response preparation and initial orienting to environmental input may require substantial processing capacity -- and thus be potentially prone to the influences of stress. A mechanism for this disruption may be related to the finding that correct performance was associated with a substantial allocation of capacity.

#### 8. Principal Components Analysis of Cardiac Inter-beat-interval During a Performance Task

Traditional analyses of physiological data involve feature extraction and scoring followed by an analysis of the central tendency, e.g., arithmetic mean, of these extracted features. Examination of the variance of a physiological response and its covariance during resting and stimulated conditions is an exception rather than the rule (compare however, the section on complex demodulation in the progress report for work unit 125). Over the last two years we have been exploring the application of principal components analysis to physiological responses, in particular to evoked cortical and cardiac responses. Principal components analysis has a number of desirable features: a) original data are the input; b) statistically independent components reflecting features in the data are extracted; c) in most cases, original data can be adequately described by a smaller number of components than the number of original data points; and d) expressing the original data as cross-products, covariances, or correlations allows the assessment of respectively, mean, variance, and covariance simultaneously; variance and covariance; and finally covariance alone with the influence of mean and variance removed. Our use of the techniques follows most closely that of Tucker (33). General treatments are found in Glaser & Ruchkin (5).

The seven beat sequence from the study of cardiac cycle and speed-accuracy tradeoff were analyzed using principal components analysis (Publication 2). This analysis produced three components which accounted for over 85% of the variance and were readily identifiable as corresponding to the initial (INIT), middle (MID), and late (LATE) beats within the seven beat sequence. Figure 5 shows the pattern of weighted principal component loadings as well as the mean waveform of the original data. Essentially identical results were obtained over the eight independent replications of the analysis which corresponded to the eight volunteers serving in the experiment. The principal component scores were analyzed to assess their sensitivity to the experimental manipulations of speed emphasis and cardiac cycle time. These results showed that both the Mid and Late components were consistently altered by the speed emphasis conditions. Further analysis suggested that perceptual influences on the Mid component were independent of influences on the Late component. The Late component seemed related to the motor response

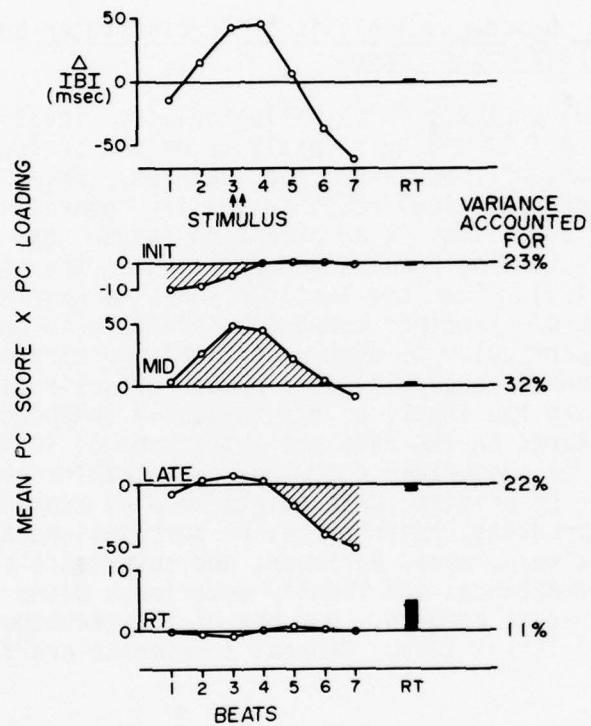


Figure 5. Principal component solution for seven inter-beat-intervals concurrent with a reaction time task.

to the choice stimulus. These results suggest that influence over cardiac IBI in this performance situation cannot be interpreted solely in terms of either motoric or perceptual influences.

Two further sets of data were examined in order to gain a deeper understanding of the Init, Mid, and Late components. Control data were analyzed from alert, resting volunteers not engaged in any performance task; and the data from the eight experiment volunteers was re-analyzed using random starting points for the seven beat sequence rather than the third beat prior to the choice stimulus. Principal components analysis of both sets of control data produced clear, consistent Init, Mid, and Late components. Thus, the Init, Mid, and Late components seem to reflect stable dependencies between beats that are reliably extracted when covariances are examined using a seven beat window. A close examination of the randomized and experimental components suggested that the task varied the covariance structure somewhat. Although tantalizing, this result was not sufficiently clear to interpret without further empirical work.

Use of the principal components analysis has provided a clear description of the strong, inherent organization of cardiac control of IBI. Psychophysiological effects may well be expressed within the context of this organization, rather than by inserting a completely independent response form. Further value gained through the analysis was the capability of separating and analyzing statistically independent components of the IBI response. This capability allowed relatively clear conclusions without the confounding influences of beat to beat dependencies and subjective feature identification.

Project 3E762771A804 MILITARY PSYCHIATRY

Work Unit 043 Military stress: Health, performance and injury factors

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3. DATE PREV SURVEY 76 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY <sup>a</sup> U	6. WORK SECURITY <sup>a</sup> U	7. REGADING <sup>b</sup> NA	8. DSBPN INSTN <sup>b</sup> NA	9a. SPECIFIC DATA- CONTRACTOR ACCESS <input type="checkbox"/> YES <input type="checkbox"/> NO
10. NO./CODES: <sup>a</sup> a. PRIMARY 62771A	PROGRAM ELEMENT 3B762771A804	PROJECT NUMBER 00		TASK AREA NUMBER 004	10. LEVEL OF SUM a. WORK UNIT	
b. CONTRIBUTING	c. CONTRIBUTING CARDS 114F					
11. TITLE (Pecode with Security Classification Code) <b>(U) Neuroendocrine Response to Military Stress</b>						
12. SCIENTIFIC AND TECHNOLOGICAL AREA <sup>a</sup> 012600 Pharmacology 002300 Biochemistry 016200 Stress Physiology 003500 Clinical Medicine						
13. START DATE 76 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD In-House			
17. CONTRACT/GRANT a. DATES/EFFECTIVE: N/A	EXPIRATION:	18. RESOURCES ESTIMATE PRECEDING	19. PROFESSIONAL MAN YRS 77	20. FUNDS (In thousands) 450		
b. NUMBER: <sup>a</sup>		FISCAL YEAR	CURRENT	430		
c. TYPE:	d. AMOUNT:					
e. KIND OF AWARD:	f. CUM. AMT.					
21. RESPONSIBLE DOD ORGANIZATION NAME: Walter Reed Army Institute of Research Washington, D.C. 20012 ADDRESS: <sup>a</sup>	22. PERFORMING ORGANIZATION NAME: Walter Reed Army Institute of Research Division of Neuropsychiatry ADDRESS: Washington, D.C. 20012	PRINCIPAL INVESTIGATOR (Pecode NAME IF U.S. Academic Institution) NAME: Meyerhoff, JL, MD TELEPHONE: (202) 576-3559 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]				
23. GENERAL USE Foreign Intelligence Not Considered	24. ASSOCIATE INVESTIGATORS NAME: Mougey EH NAME: Holaday JW					
25. KEYWORDS (Pecode EACH with Security Classification Code) <b>(U) stress (U) transmeridian desynchronization (U) neurotransmitters (U) hormones</b>						
26. TECHNICAL OBJECTIVE, <sup>a</sup> 27. APPROACH, 28. PROGRESS (Pecode individual paragraphs identified by number. Pecode text of each with Security Classification Code.) P3. (U) To examine neuroendocrine and neurochemical correlates of stressors specific to the military environment. Types of stress to be studied will include extremes of heat and cold, psychological stress, as well as desynchronization of circadian rhythm. P4. (U) Laboratory and field studies will examine the neuroendocrine response to psychological stressors (i.e., simulated combat exercises) ambient temperature extremes and transmeridian desynchronization. These responses will be correlated with simultaneously-obtained data on performance decrement in the same subjects. Hormonal responses will provide bases for inferences concerning central nervous system neurotransmitter pathways essential to adaptation to stress. This information is used to recommend pharmacologic and other therapies. P5. (U) 76 10 - 77 09 We have found that endocrine responses to stress vary with the type of stressor. While forced immobilization elevates plasma corticosteroids as effectively as cold stress, the former produces a rapid ten-fold elevation in plasma prolactin, compared to a mere doubling following exposure to cold. A series of studies was completed which describes secretory rates and circadian patterns of cortisol secretion during both basal and ACTH-stimulated conditions. In addition to a marked circadian rhythm, the studies revealed an ultradian with a periodicity of 80-90 minutes which was relatively independent of ACTH. Additional studies suggest a role for pituitary beta-lipotropic hormone, 61-91 subunit, in adaptation to environmental stress. These effects are antagonized by the hypothalamic peptide, thyrotropin releasing hormone. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.						
1484						
<small>*Available to contractors upon originator's approval.</small> DD FORM 1 MAR 68 1498 <small>PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.</small>						

Project 3E762771A804 MILITARY PSYCHIATRY

Work Unit 044 Neuroendocrine Response to Military Stress

Investigators.

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E. H. Mougey, M.S.; J. W. Holaday, Ph.D.; D. R. Collins  
B.S.; L. L. Pennington, B.S.

I. Endocrine Profile as Indicator of Stress Response.

A data base sufficient to design preventive and therapeutic measures must include study of internal as well as environmental factors which mediate the resistance to neuropsychiatric breakdown in combat. This is a multidisciplinary effort addressing the development and use of laboratory models to define and describe the spectrum of pathological responses and disease states caused by stressors in the military environment, a continuum extending from total breakdown through psychosomatic diseases, to subtle decrements in performance. Stress may be viewed in terms of direct effects on the brain, in terms of effects on the brain's control of internal organs (i.e., hypertension, ulcers, colitis, etc.), or in terms of effects on performance. The likelihood of a given stressful situation producing symptoms of psychiatric disability can be increased by pre-existing conditions such as chronic fatigue. Efforts to prevent or treat stress-induced disability and to improve military performance depend in part on detailed knowledge about the brain systems which address the soldier's "internal" and "external" worlds and how those brain systems interact. Understanding of these internal factors often requires laboratory experiments which pursue leads developed in field studies and support work in the Departments of Military Psychiatry and Military Medical Psychophysiology. Hormonal response to stressors, for example, which can be observed in both field and laboratory studies, provides a critical link in facilitating coordination between those two efforts. Studies of neurochemical, neurophysiological and neuroanatomical regulation of the body's response to stress provide a means of interpreting the field data.

Emphasis is placed on considering total patterns of neuroendocrine response rather than on the potentially misleading study of individual neuroendocrine systems. A major immediate goal is to define in detail the characteristic hormone response profiles for various stressful stimuli: effect of multiple stressors, environmental factors such as continuous performance requirements, transmeridian desynchronization and/or ambient temperature, biological factors such as differences in sex or age, or effects of pain or neural injury. Rational design of prevention and treatment regimens for neuropsychiatric illness induced

by military stress requires interpretation of the hormonal response profiles for stress in terms of the neurochemical systems producing the response. Hormone profiles are measured in clinical and animal studies as indicators of stress; they provide a basis for inferences about changes in brain function. For example, plasma prolactin levels provide a sensitive indication of the degree of central dopaminergic blockade induced by antipsychotic medication (1). Growth hormone secretion is stimulated by central catecholaminergic systems and is markedly increased during slow wave sleep (2,3,4). Thus, biochemical measurements may provide a useful measure of adequacy of rest periods during continuous performance studies. Additional neurochemical studies in animals will directly examine effects of stress in neurochemical activity of the brain and explore physiological, dietary, and pharmacologic methods for preventing stress-induced psychologic and physiologic disease.

Mission emphasis on continuous performance requires closer attention to biological rhythms in stress indices. Accordingly we have examined circadian rhythms, ultradian rhythms and rates of cortisol secretion. Cortisol secretory patterns were studied in two chair-adapted monkeys by simultaneous measurement of plasma  $^{14}\text{C}$  concentration and specific activity of cortisol after an iv bolus of  $^{14}\text{C}$  labeled hormone. The results indicated that fluctuating plasma cortisol concentrations are the result of episodic secretion by the adrenal cortex. Specific activity changes during these spontaneous secretory bursts indicated occasional submaximal activity. In addition, cortisol secretory rates calculated during basal (20.4  $\mu\text{g}/\text{min}$ ) and ACTH-stimulated (28.4  $\mu\text{g}/\text{min}$ ) conditions in a total of seven monkeys were significantly different ( $p < 0.05$ ), further demonstrating that spontaneous secretory bursts were usually submaximal. From plasma samples collected at 10 min intervals, a cortisol distribution  $t_{1/2}$  of 6 min and a clearance  $t_{1/2}$  of 66 min were found. The apparent volume of distribution for this hormone was 4.8 liters, a value far in excess of extracellular fluid volume estimates. The circadian pattern of plasma cortisol in these monkeys resembled that reported for man, but monkeys had twice as many episodic bursts and over twice the mean cortisol levels as man. However, the 24 h production rate was 10.5 mg, a value within the range of human production. A highly synchronized ultradian cortisol rhythm with a predominant periodicity of 85-90 min was observed in studies of eight isolated monkeys. The persistence of this rhythm during ACTH infusions suggests that feedback is not causative and that bursts of cortisol secretion may not always be dependent on preceding ACTH release. Furthermore, ultradian cortisol rhythms may be harmonically related to the circadian rhythm.

We recently developed a radioimmunoassay for urinary tetrahydrocortisol (THF). This was evaluated for use as a routine screening test for stress effects in humans and experimental animals. Urine samples collected from monkeys which were subjected to acute immobilization stress were assayed for total and free THF, total and free cortisol and total 17OHCS. All radioimmunoassayed samples for THF and cortisol were purified by TLC to check for specificity of the antibodies used. The

total THF assay correlated very well with the 17OHCS measurement ( $r = .96$ ) and showed a high degree of specificity. The TLC purified value was approximately 90% of the initial value. All compounds measured showed increases characteristic of the enhanced adrenocortical activity in stressed monkeys but their sensitivity as stress indices varied. The urinary 24-hr free cortisol value was the most sensitive, increasing 43-fold during acute immobilization, while total urinary THF increased 9-fold and total urinary 17-hydroxycorticosteroids increased 6-fold.

We prepared our own antibody to prednisolone-3-BSA conjugate. This was evaluated by comparison with a commercially available antibody to cortisol-21-BSA. The antibody was found to be specific enough for routine cortisol assay thus eliminating the need to purchase the expensive antibody commercially. Studies were conducted to determine if microwaved rat plasma samples could be assayed for total thyroxine by competitive protein binding. Recovery studies showed that added T<sub>4</sub> was not being completely recovered. This was attributed to either the destruction of the hormone or interference in the binding assay by artifacts released by the blood as a result of heating during microwave treatment or to irreversible binding to proteins or other materials in the plasma.

A new and increasingly important area of research concerns the role of pituitary and brain peptide hormones in adaptation to environmental stressors. Most promising are peptide subunits of beta lipotropic hormone ( $\beta$ LPH).  $\beta$ LPH (MW 6900) was isolated from pituitary glands and characterized by Li in 1964 (5). Its major function was thought to be lipolysis and liberation of free non-esterified fatty acids. Recently, however, interest has focussed on a  $\beta$ LPH peptide fragment composed of amino acids 31 through 91 of  $\beta$ LPH. This peptide has been named Beta-endorphin ( $\beta$ E). It is a very potent analgetic and hypothermic agent. Immunocytochemical studies have demonstrated intense staining for both  $\alpha$  endorphin ( $\alpha$ E) and  $\beta$  endorphin ( $\beta$ E) in the pars intermedia of the rat pituitary, with discrete cells staining positively in the anterior pituitary as well and with no staining in the pars nervosa (6). While the  $\alpha$  endorphin antibody was highly specific for that peptide, the  $\beta$  endorphin antibody cross-reacted considerably with  $\beta$  lipotropic hormone ( $\beta$ LPH), which was previously demonstrated to be present in the pars intermedia and pars distalis (7). The function of the pars intermedia is not known with certainty. It has been associated with melanocyte stimulating hormone (MSH) (8), adrenocortical stimulating hormone (ACTH) (7) as well as  $\beta$ LPH (7). We are interested in assessing the role of the pituitary as the source of circulating endorphins and to ascertain the interrelationship among endorphins and hypothalamic, pituitary, and adrenal hormones. In studies of hypophysectomized and adrenalectomized rodents, we have determined that both surgical procedures significantly enhance the apparent potency of  $\beta$  Endorphin in tests for antinociception, catalepsy, and altered body temperature. Further results have shown that corticosteroids and thyrotropin releasing hormone (TRH) antagonize many of the effects of  $\beta$  Endorphin. Most recently, our heat-stress experiments have provided the first physiological evidence implicating the

endorphins in adaptation to acutely and chronically elevated temperatures. Other work (9) suggests that  $\beta$  endorphins ( $\beta$ LPH amino acid segment 61-76) have some of the same behavioral effects as  $\beta$  Endorphin ( $\beta$ LPH amino acid segment 61-91). In summary, we know that endorphins are found in the pituitary gland and in brain, they possess antinociceptive activity and we believe they may be involved in thermoregulation as well. Several important questions remain to be addressed. Can endorphins be measured in blood? Are they released from the pituitary? If so, what regulates their release? What are the physiological and psychological roles of the endorphins, if any? The potential relevance of these questions to military medicine is illustrated by the data of Beecher (10) who studied the management of pain in soldiers wounded in combat in WWII. Of 215 severely wounded soldiers, 32.1% reported no pain and 25.6% only slight pain.

Project 3E762771A804 MILITARY PSYCHIATRY

Work Unit 044 Neuroendocrine Response to Military Stress

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION <sup>9</sup>	2. DATE OF SUMMARY <sup>9</sup>	REPORT CONTROL SYMBOL	
3. DATE PREV SURVEY	4. KIND OF SUMMARY	5. SUMMARY SEC <sup>9</sup>	6. WORK SECURITY <sup>9</sup>	DAOC 6455	77 10 01	DD-DR&E(AR)636	
76 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES	<input type="checkbox"/> NO
10. NO./CODES: <sup>9</sup>	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	62771A	3E62771A804		00	045		
b. CONTRIBUTING							
c. CONTRIBUTING	Cards 114F						
11. TITLE (Precede with Security Classification Code) <sup>9</sup>							
(U) Follow-up Studies of Human Volunteers who received Psychoactive Substances							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS <sup>9</sup>							
003500 Clinical Medicine 013400 Psychology							
13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD			
76 07	CONT	DA		C. In-House			
17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN-YRS			
a. DATES/EFFECTIVE:	N/A	EXPIRATION:	FISCAL	8	20. FUNDS (In thousands)		
b. NUMBER: <sup>9</sup>		YEAR	77	464			
c. TYPE:			78	0			
d. KIND OF AWARD:		e. AMOUNT:					
19. RESPONSIBLE DOD ORGANIZATION		20. PERFORMING ORGANIZATION		NAME: Walter Reed Army Institute of Research Division of Neuropsychiatry ADDRESS: Washington, DC 20012			
NAME: Walter Reed Army Institute of Research Washington, DC 20012 ADDRESS: <sup>9</sup>							
RESPONSIBLE INDIVIDUAL NAME: Raptmund, COL G. TELEPHONE: (202) 576-3551				PRINCIPAL INVESTIGATOR (Punish SICAN II U.S. Academic Institution) NAME: Marlowe, D.H., Ph.D. TELEPHONE: (301) 427-5210 SOCIAL SECURITY ACCOUNT NUMBER: [REDACTED]			
21. GENERAL USE Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS NAME: Garcia, LTC J. NAME: Redmond, MAJ D.			
22. KEYWORDS (Precede EACH with Security Classification Code) <sup>9</sup>							
(U) Lysergic Acid Diethylamine; (U) Epidemiology; (U) Clinical Sequelae							
23. TECHNICAL OBJECTIVE, <sup>9</sup> 24. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) In the period of 1956-1969 some 600 military volunteers received LSD in the course of experiments with chemical warfare agents carried out at Edgewood Arsenal AMC. The purpose of this work unit, mandated by the Surgeon General, has been a follow-up medical evaluation to determine whether there have been any clinical or other sequelae to the ingestion of this agent.							
24. (U) A medical and epidemiological follow-up examination and study has been designed utilizing the various disciplines of clinical medicine, psychiatry, psychology and epidemiology.							
25. (U) 76 10-77 09 This organization is responsible for the design of the medical evaluation, the monitoring of data gathering and analysis and evaluation of data. All of the initial pilot medical examinations have been completed and the final examination module has been accepted by the Office of the Surgeon General for use by a HSC medical center. The final set of examinations for all former subjects is anticipated to begin in the early part of this fiscal year. A central data file has been established. The final forms of the various instruments have been completed as has the initial system for analysis of the data. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 76 - 30 Sep 77.							

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1 MAR 68

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AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3E762771A804

Work Unit 045 Follow Up Studies of Human Volunteers Who Received Psychoactive Substances

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Description

During the period 1956-1969 some 600 military volunteers received Lysergic Acid Diethylamide (LSD) in the course of experiments carried out at Edgewood Arsenal, AMC. In 1975 OTSG mandated that the WRAIR Division of Neuropsychiatry take responsibility for the development of a follow up medical evaluation of these individuals. The Division of Neuropsychiatry was also charged with assessing whether or not a scientific study was feasible that would determine if there were specific clinical sequelae causally related to the ingestion of LSD under the auspices of researchers in chemical warfare.

Progress

An initial pilot study was completed in 1976 (Project 28) and a medical follow-up examination and evaluation module designed by this organization. A first pilot study (Project 50/50) was carried out in FY 77. The follow up examinations were given by the staff of WRGH utilizing the examination module developed at WRAIR. It had been the intention, during this pilot phase, to evaluate 50 former volunteers and fifty matched controls. Controls were considered to be of critical importance to a valid study particularly since there is a lack of baseline clinical data in the records of the volunteer participants. It soon became evident that the technical problems involved in finding matched controls for such a retrospective study were almost insurmountable and that a matched control study was not feasible. All former LSD recipients

and no scientifically viable way could be developed to determine whether or not health problems presented by the patients were in actuality rooted in past LSD ingestion. Preliminary analysis of the available data was carried out and recommendations were made to the Surgeon General during a briefing in July 1977 as to the options for evaluating the remaining LSD recipients.

Project 3E762771A805

MICROWAVE INJURY PREVENTION AND TREATMENT

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION# DA OB 6484	2. DATE OF SUMMARY# 77 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUMMARY 76 10 01	4. KIND OF SUMMARY D Change	5. SUMMARY SECY# U	6. WORK SECURITY# U	7. REGRADING# NA	8. DISSEM INSTRN# NL	9. SPECIFIC DATA- CONTRACTOR ACCESS <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
10. NO. /CODES: a. PRIMARY 62771A	PROGRAM ELEMENT PROJECT NUMBER 3E762771A805	TASK AREA NUMBER 00		WORK UNIT NUMBER 041		
b. CONTRIBUTING						
c. CONTRIBUTING						
11. TITLE (Precede with Security Classification Code) Biological Interactions with and Hazards of Microwave Radiation						
12. SCIENTIFIC AND TECHNOLOGICAL AREAS# 014100 Radiobiol 012900 Physiol 014000 Rad Chem 017000 Wave Prop 013400 Psychology						
13. START DATE 71 07	14. ESTIMATED COMPLETION DATE CONT	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House			
17. CONTRACT/GANT		18. RESOURCES ESTIMATE				
a. DATES/EFFECTIVE: NA	EXPIRATION:	FISCAL YEAR	PRECEDING CURRENT	b. PROFESSIONAL MAN YRS 6	d. FUNDS (in thousands) 684	
b. NUMBER: -		78		7	702	
c. TYPE: -						
d. AMOUNT: -						
e. KIND OF AWARD: -	f. CUM. AMT. -					
19. RESPONSIBLE DOD ORGANIZATION		20. PERFORMING ORGANIZATION				
NAME: Walter Reed Army Institute of Research Walter Reed Army Medical Center ADDRESS: Washington, D.C. 20012		NAME: Walter Reed Army Institute of Research Dept of Microwave Research ADDRESS: Div of Neuropsychiatry Walter Reed Army Medical Center Washington, D.C. 20012 PRINCIPAL INVESTIGATOR (Name if U.S. Academic Institution) Larsen, L.E. NAME: 202-576-3638 TELEPHONE: [REDACTED]				
RESPONSIBLE INDIVIDUAL NAME: Rapmund, COL. G. TELEPHONE: 202-576-3551		SOCIAL SECURITY ACCOUNT NUMBER ASSOCIATE INVESTIGATORS NAME: Jacobi, J.H. NAME: Hunt, E.L.				
21. GENERAL USE Foreign intelligence not considered						
22. KEYWORDS (Precede EACH with Security Classification Code) (U) Microwave Hazards; (U) Biophysics; (U) Dosimetry; (U) Bioeffects; (U) Military Medicine; (U) Psychology						
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Punish individual paragraphs identified by number. Precede text of each with Security Classification Code)						
23. (U) To provide technical and medical information to the Surgeon General, system developers and agencies responsible for safety standards in order to protect the health and effectiveness of military units and affected civilian populations in microwave and RF environments. This requires analysis of the biophysics and bioeffects attributable to non-ionizing radiation under laboratory conditions which reasonably simulate operational exposures.						
24. (U) To perform basic and applied research on the problem of microwave and RF interactions with biosystems at all levels of analysis from the cellular and molecular to metazoan physiology, pathophysiology and behavior. This requires development of measurement systems for dosimetric analysis ex vacuo, in vitro, and in situ; the evaluation of frequency, power level, polarization and modulation as important parameters of the radiation; and the use of low level energy to assess the functional state of cells and tissues.						
25. (U) 76 10 - 77 09 Progress has included the demonstration of feasibility for non-invasive microwave dosimetry using scattering parameters and time delay spectrometry; development of network analysis methods for high speed, broad band measurement of permittivity in biological tissues, development of methods for improved spatial resolution in microwave images of modeled biological targets; preliminary studies to assess functional states of cells and molecules in vitro by electromagnetic analysis; and development of microwave transparent electrodes for temperature measurement in situ. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 76 - 30 Sep 77.						
1602						
Available to contractors upon originator's approval.						
DD FORM 1 MAR 68 1498		PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.				

Project 3E762771A805 MICROWAVE INJURY PREVENTION AND TREATMENT

Work Unit 041 Biological interactions with and hazards of microwave radiation

Investigators.

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B.S.

I. Introduction

The Department of Army is a major consumer and developer of communications and electronic systems which employ a large segment of the electromagnetic spectrum. The frequencies actually employed extend from the audio band (in the order of  $10^3$  Hz) to K band (in the order of  $10^{11}$  Hz). In terms of total pieces of deployed equipments, the numerically most prominent band is the HF band ( $10^6$  to  $10^9$  Hz) where nearly one-quarter of a million sources exist (1). These equipments are primarily tactical communications devices and are typically of relatively low radiated power (in the order of  $10^{-1}$  W). The most prominent high power band (in the order of  $10^3$  to  $10^5$  W) is the radar L band (in the order of  $10^9$  Hz) where approximately 200 sources exist in CONUS alone. A typical application for these sources is deployment as air defense radars, i.e. target acquisition and tracking radars. The band of most active development is radar K band (in the order of  $10^9$  to  $10^{11}$  Hz). Typical uses for these sources are as terrain mapping and guidance radars. Total radiated power is most often moderate, in the order of  $10^2$  to  $10^3$  W.

Biological effects are definitely known to extend from 34 MHz to 22 GHz. The lower frequency extreme ( $34 \times 10^6$  Hz) represents the frequency of optimal energy transfer to a grounded 1.7 M man with long axis parallel to the electric field. The upper frequency extreme ( $22 \times 10^{11}$  Hz) represents the relaxation frequency of free water. There are a myriad of factors that come into play at these and intermediate frequencies. Prominent among these is depth of penetration, coupling efficiency and focusing factors in both the transmitted field and the geometry of target organs. It is likely that frequencies beyond the two extremes described above will prove to be important, especially in scientific application.

The Surgeon General of the United States Army has direct responsibility under AR 70-1 for the final determination of operator and personnel safety in the presence of these emitters. The Surgeon General's resources for this task are chiefly USAEHA and USAMRDC. The enterprise is greatly complicated by the fact that no international agreement exists on the standards of safe exposure. This lack of agreement is the result of many factors, not all of them scientific. There are, however, numerous discrepancies of fact, not to mention interpretation, that exist within Western scientific circles which are sufficient to engage our attention. Prominent among these are the role of frequency dependent bioeffects, the

role of modulation parameters, and the role of combined stressors in the form of multiple simultaneous emitters as electronic warfare continues to grow faster than any other area of material development. Thus, the role of EMR in the modern battlefield represents a prodigious problem for which a paucity of objective, relevant scientific information exists to assist a rational management of the Army Medical Department's responsibilities.

A major fraction of the task resides within the Medical R&D Command since even if detailed descriptions of all emitters under all circumstances of use did exist, we would still be unable to defend by objective means a medical evaluation of the risk and hazards presented by all or even a large portion of the conditions likely to be present on the modern battlefield. This already difficult situation is further complicated by the fact that adversary forces employ emitters which are obviously not under our control, and that the CE/EW environment must be considered as an element in the event that adversary forces deploy CBW or directed energy weapons.

Finally, there is the potential that diagnostic, therapeutic and scientific applications exist for the use of RF and microwave energy. This possibility does not contradict the role of hazards research. Past experience offers many examples where beneficial properties of agents were exploited only after they were first identified by their hazards. This includes a number of "poisons" also used in chemotherapies; and, of course, x-ray which has proven carcinogenic properties. Indeed, the history of medicine often suggests that one could infer useful actions only after biologic activity was confirmed by toxicity.

The subject which this department is charged to address is, therefore, one of bewildering complexity. We must consider a vast range of frequencies, power levels, modulation parameters and multiple sites of action involving all organ systems. The pressing question is how to begin.

We do have considerable experience to guide a research plan. The Triservices EMR project spanned a period of nearly 3 decades. Yet the lack of reproducible results have limited any generality that this period of activity could bring to bear on today's problems. Part of the explanation for this shortage is the lack of adequate instrumentation for quantification of absorbed dose. As a result, this department has many major program elements directed toward dosimetry. These include programs for development of implantable, microwave transparent electrodes for temperature measurement, calorimetry, and dosimetric analysis by radiofrequency tomography as detailed in the project summaries to follow. A corollary to this aspect of the research program is its contrast to earlier thought where internal field measurements were regarded as "senseless".

"Standing wave pattern in the body make it senseless to talk about energy flux in the body and to use it as a measure of dosage. All

dose or dose rate statements must refer to field or flux values in the distant field defined sufficiently far from the body to be affected by its presence (2)."

Another element is our continued interest in measurement of the electromagnetic constitutive parameters of tissues under physiological conditions. It is abundantly clear that permittivity measurements in autopsy material at room temperature neglect target organ geometry, physiological state, and any functional responses to the radiation (of special importance in this regard is the role of local vasodilation in response to local hyperthermia). Many elements of department program are directed to this problem. Prominent among these are the in situ permittivity, cellular and molecular programs (cf. program summaries). It is important to realize that this represents a significant departure from prior thought typified by the conclusion published in the 1970 Symposium Proceedings on the Biological Effects & Health Implications of Microwave Radiation from which I quote: "no further work is needed in regard to absorption coefficients (3)."

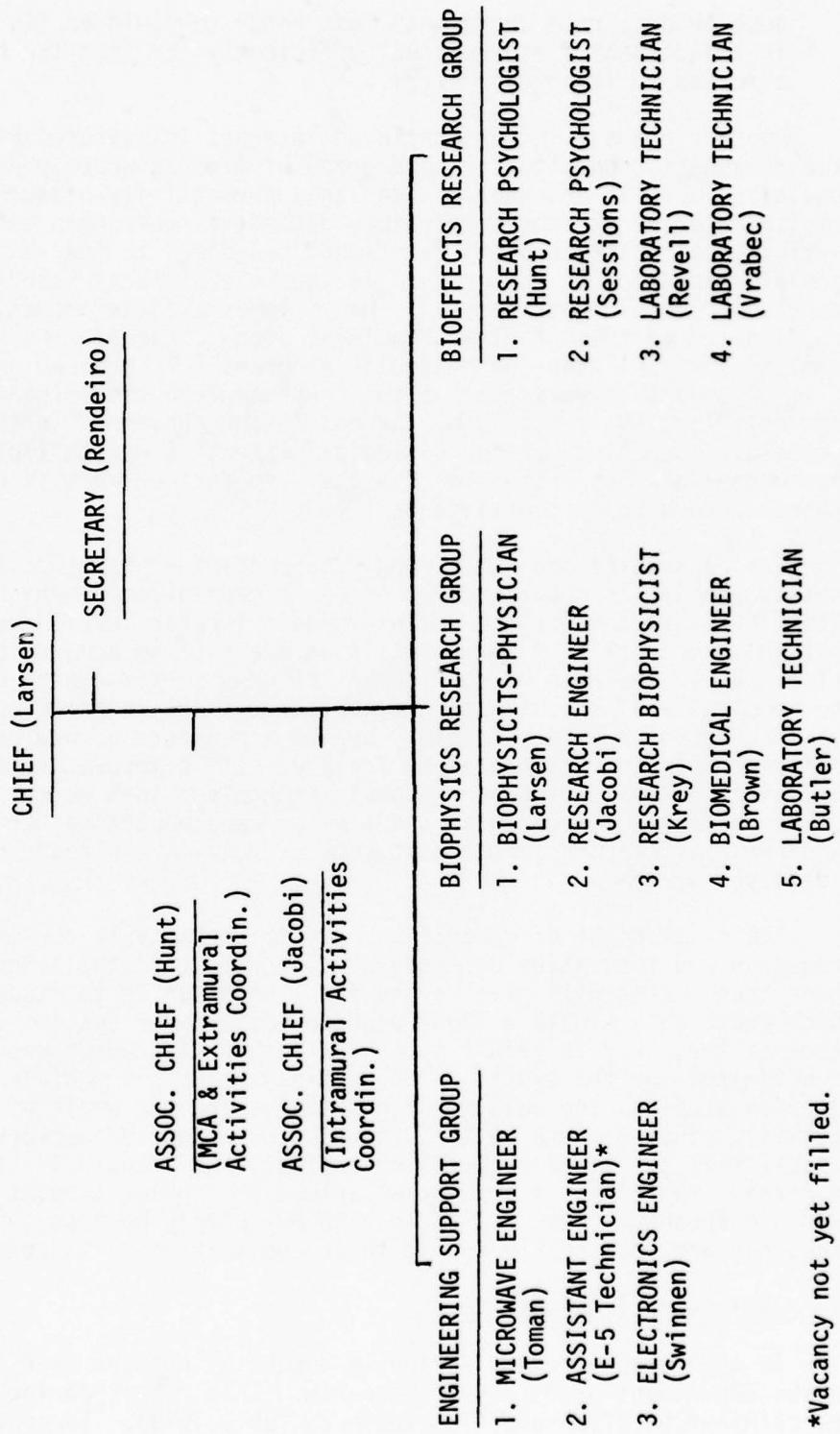
The department program strongly supports the view that it is important to acquire an understanding of basic mechanisms by which EMR interacts with biosystems at the cellular and molecular level. We believe that this approach is fundamental if we are ever to acquire predictive value. Given the rate of development of Army systems which use EMR and the complexity of the situation described in earlier paragraphs, a research approach directed solely by the appearance of new systems in the equipment inventory is doomed to failure. It is imperative to acquire sufficient understanding of the basic mechanisms that we can contribute to EMR parameter selection at the time of development rather than recommend remedial measures after vast sums of money are already committed to a deployed system.

The final point of general scientific strategy is the importance of frequency and modulation parameters. The recent installation of a high power transmitter will give us the first opportunity to study fields which reasonably simulate those produced by a radar system. The importance of frequency is illustrated in a number of studies where frequency is optimized for the system under examination. This includes the taste aversion studies, the cell membrane studies and the whole of the permittivity studies where we are extending our range of network analysis capabilities to extend from  $10^3$  Hz to  $18 \times 10^3$  Hz. Again it is important to realize that this is a major departure from prior thought whereby the exposure frequency was most often 2450 MHz simply because tubes at this frequency are less costly due to their application in industrial heating.

## II. Administrative Activities

In the present report period, a number of changes have taken place in the Department of Microwave Research. This report period began with the culmination of a reduction-in-force (July 1976). Several people in

DEPARTMENT OF MICROWAVE RESEARCH, WRAIR  
 Interim Organization (Construction Period)



the department were affected. Three members of the previous staff were lost. An electronic engineer (Mr. Swinnen), a biophysicist (Mrs. Krey) and a biomedical engineer (Mr. Brown) were acquired. In addition, a microwave engineer (Dr. Toman) was recruited. Subsequently, a department reorganization (see attached organization sheet) occurred in which the intramural and extramural management functions were distributed into staff positions rather than the directorate system which previously existed (effective date, 27 July 1977). In addition, the investigators and support personnel were grouped into pools based along functional lines that may be applied to research tasks which proceed from mission requirements. Concurrently, a comprehensive departmental research program has been formulated and submitted through the Division of Neuropsychiatry to the Director, WRAIR for approval. This research plan contains a major expansion in the cellular and molecular aspects of the program. This expansion will be evident in the project summaries presented below.

The department responded to inquiries directed to the Army Medical Department via the Office of the Director of Defense Research and Engineering from the United States Senate Committee on Commerce, Science and Technology radiation oversight hearings (July, 1977). The basic elements of research strategy, scientific management and program structure were presented in open testimony with other DOD agencies.

In this report period a major item of capital equipment was installed: the Cober High Powered Microwave Transmitter. This transmitter contains a 400 KW power supply and could effectively operate pulse magnetrons in the megawatt range of peak power output. Presently, it is fitted with a 12 KW (CW) X band klystron. No experiments with this new transmitter were performed during the present report period.

In addition, a contract was let on 10 August 77 for reconstruction of Buildings 502/503 where the department laboratories and offices are located. Coincident with this, several laboratories were relocated in temporary quarters. In this turmoil, only one investigator has suspended research activities, and this was occasioned by the need to attend AMEDD Advanced Officer Training for six months.

### III. Research Activities: Intramural Program

#### A. Non-invasive Microwave Interrogation of Dielectric Objects Part I: By Scattering Parameters (L.E. Larsen and J. H. Jacobi)

A project was undertaken in the 1975 report period to initiate feasibility studies for microwave Dosimetric Analysis by Radiofrequency Tomography (DART). Since that time significant progress has been made to confirm the feasibility of microwave imagery. Specifically, two papers were presented at the International URSI/IEEE Antennas & Propagation Symposium in October 1976. The first of these is abstracted below:

"Incident microwave radiation may be used to interrogate the physi-

cal structure and RF electrical properties of dielectric objects. It would appear to be possible that such radiation could be used to image biological targets on the basis of these properties which are not available to x-ray, ultrasonic or nucleonic interrogation. Indeed, the properties used to form a microwave image may be uniquely relevant to the structure and function of biological systems.

Single and multiple dielectric discontinuities of various geometries and magnitudes were investigated in a medium of deionized water. Mechanical scanning permitted the investigation of spatial resolution and aperture effects.

The scattering parameters measured were the amplitude and phase of  $S_{11}$  and  $S_{21}$  for a phase locked 3243 MHz source. These were studied as functions of space for the various dielectric discontinuities presented. Dielectrically loaded, matched antennas were incorporated into a digitally controlled scanner which was interfaced to an automatic network analyzer (HP 8243A). The resulting complex functions of space were further processed to create a real valued function of space for intensity presentation of the data. The real valued spatial series were also adverse filtered by digital methods to compensate for antenna patterns.

Use of this system allowed the detection of single and multiple dielectric discontinuities of dimensions well below one wavelength in water. The results were sensitive to aperture size, wavelength, and antenna separation."

Since that time, a new scanning system and new antennas have been developed. The spatial resolution and mechanical accuracy of the system have been improved with the development of water coupled S band antennas and a computer controlled antenna scanner based on the use of optical bench components, respectively. These antennas provide greater aperture area reduction due to the higher dielectric constant of the medium (81 as compared to 16), broader bandwidth (VSWR < 1.4 over most of the 2 to 4 GHz operating range), and improved freedom from multipath. This new antenna was applied to a target of greater complexity than those reported at URSI last October. The target consisted of a block of phantom fat 10 cm long, 2 cm high and 1 cm deep in which two water filled 1.8 mm capillarity tubes were imbedded at middepth and separated by 11 mm. Reflection ( $S_{11}$ ) and transmission ( $S_{21}$ ) scattering parameters were measured with this complex target submerged in water at the midpoint between the two antennas.

Both  $S_{11}$  and  $S_{21}$  clearly detected the two capillarity tubes under 3 layers of dielectrics. The spacing of the two tubes could be accurately inferred from the peaks of  $|S_{11}|$  and the valleys in  $|S_{21}|$ . On the basis of these results, we estimate the present scanner will reach the Rayleigh criterion for resolution at about 5 mm.

Future directions in this project will include studies for further

improvements in spatial resolution by signal processing antennas and image formation by digital implementation of lens functions with the complex spatial series which represent the reflected and transmitted fields.

B. Microwave Interrogation of Dielectric Objects

Part II: Microwave Time Delay Spectroscopy (J.H. Jacobi and L.E. Larsen)

In the present report period, development was continued on Microwave Time Delay Spectroscopy as a technique for non-invasive interrogation of biological targets. As a review of first principles, this is a method for measuring propagation delay through a biological sample by illuminating it with a frequency swept microwave signal. The propagation delay is estimated by measurement of the instantaneous frequency difference between the signal that propagates through the target and a signal that propagates through a path with fixed delay. In these experiments, the signal was swept from 2 GHz to 4 GHz in 16 msec and constant  $df/dt$ . The first realization of this system and early experimental results were presented to the International URSI/IEEE Antenna & Propagation Symposium in October 1976. This report is abstracted below:

"Dielectric materials have the property that the velocity of propagation of electromagnetic energy in the material is a function of the dielectric constant of the medium. This property may be exploited to permit mapping of dielectric inhomogeneities in a target according to the time delays present in the signal which passed through the target. A method is described whereby time and transmitted frequency are related with a linear chirp. The received signal is mixed with a reference signal which does not pass through the target. Time delays due to changes in velocity of propagation, path length effects due to diffraction, and calibration effects appear as frequency shifts in the mixer output. A sweep oscillator is chirped from 2.0 to 4.0 GHz, and the mixer output is processed by generalized harmonic analysis.

The method is capable of resolving a change in path length which is equivalent to 40 picoseconds of propagation time. Time delay spectra were collected as functions of space by mechanical scanning through various dielectric discontinuities in a medium of deionized water. It was possible to detect targets with dimensions less than one wavelength in water for the highest frequency transmitted."

Since that time many significant improvements in the MTDS system were developed. Previous experiments were performed with cavity backed Archimedian spirals operating in air. This resulted in a large physical aperture (6 cm) that prevented adequate spatial resolution. Secondly, a large reflection and consequent loss of energy was introduced when the antenna was used with targets in water dominated environments.

The reflection and aperture limitations could not be solved in any

simple way. Impedance matching and simple dielectric loading would have produced a narrow band device totally unsuitable for the  $2 \times 10^7$  Hz range of the swept signal. The solution had to await the development of water coupled double ridged waveguide antenna (see separate project description). The water loaded, open-ended waveguide offered both small physical aperture (12 by 6 mm) and large bandwidth (2 GHz).

Aperture tests with a single 1.8 mm water filled capillary tube in a water tank surrounding both antennas as well as the target showed that positional changes of 2 to 4 mm were easily detected in the spectrum. This stands in marked contrast with a 30 mm figure under comparable circumstances with the previous system. Variations in the material filling the capillary tube were also detectable in the spectrum. Such changes were not measurable with the earlier system.

### C. Water Coupled Antenna (J.H. Jacobi, T. Hast and L.E. Larsen)

One of the major problems encountered in interrogation of biological targets with electromagnetic radiation in the microwave region is poor spatial resolution. When operating in the near field of an antenna the spatial resolution is monotonically related to physical aperture size. That is, if one can reduce the aperture of the sampling antenna then the spatial resolution improved.

In previous experiments, the aperture size had been reduced by loading a waveguide with solid dielectric ( $K = 16$ ) and operating in air. This produced the desired result of reducing the physical aperture area by a factor of 16 but the antenna impedance characteristics were very poor. It was well matched only over a small bandwidth. The reason for the inability to match over a large bandwidth is the existence of reflection at the air-dielectric interface at the aperture of the antenna.

After the initial experiments the solution to the impedance/bandwidth problem became clear. The entire antenna had to be immersed in a high K dielectric material to reduce the physical aperture and eliminate the air-dielectric interface. It was decided to immerse and fill an open-ended double-ridged waveguide with water. Water has a dielectric constant of approximately 77 at 3000 MHz. Immersing the antenna in water would reduce the area of the physical aperture by a factor of 77 over an air filled waveguide. On the basis of this concept and a prototype L band antenna built in our laboratory, a contract was awarded to construct a prototype S band antenna.

The prototype antenna was built and tested with very encouraging results. The VSWR of the antenna was less than 2.0 over the range from 2000 to 4000 MHz. In addition to impedance measurements, data was collected on the antenna patterns vs. frequency and separation and internal losses in the antennas. Internal loss of the antenna was considered to be of equal importance to impedance match. If all the transmitted energy were dissipated within the antenna then they would be useless. It was

found that internal losses were quite acceptable, varying to a maximum of 7 db at 4000 MHz.

In conclusion, it has been demonstrated that it is practical to reduce antenna aperture by immersing the antenna in water while maintaining good impedance characteristics and low internal losses. It is significant to note that we believe that this is the first microwave antenna to operate under water.

D. Microwave Transparent MIC Temperature Electrode (L.E. Larsen, R.A. Moore, J.H. Jacobi, F.A. Halgas and P.V. Brown)

This project made significant progress during the 1976 report period. The results of this research have been reported at the International URSI/IEEE Antennas & Propagation Symposium (Oct 1976). The findings are abstracted below:

"The microwave intergrated circuit electrode reported by Larsen, Moore and Acevedo (4) has been further developed to improve its performance: 1) It operates in significantly higher energy density fields (250 mW/cm<sup>2</sup> rather than 50 mW/cm<sup>2</sup>, CW, 2450 MHz). 2) It employs an improved resistance measurement method via four terminals located at the transducer. 3) The transducer is now glass encapsulated for improved long-term stability. 4) A hyperthin film transmission line is used for improved RF decoupling. 5) The electrode is fabricated with methods more suitable for quantity production.

The electrode employs the notion of electrothermal matching whereby the electrode segments which operate in air differ in their physical and electrical properties from those which operate in tissue. This concept requires that electrode and medium losses are comparable in order to prevent unnecessarily high source impedances and/or heat sinking from the tissue being measured. Thermographic methods were used to verify RF properties of the electrode and transmission line in simulated tissue and air.

Since that time, the temperature encoding electronics have been designed, built and tested. The thermometric properties of the electrode and encoding package have been tested for long term stability and hysteresis in a highly controlled temperature bath. The long term stability is independent of the MIC and is presently limited by the tempco of the encoding package. This error amounts to about 5 millidegrees per degree C.

Hysteresis testing over repeated 8 hour periods demonstrated no detectable hysteresis. The only variability in these tests was due to changes in room temperatures. This error contributed a maximum error of 20 millidegrees on any one run.

Future plans include further efforts to improve the tempco of the

encoder, development of an FM telemeter and implantable encoder, and development of totally flexible substrate for intraluminal use.

E. Measurement of Complex Permittivity of Biological Materials  
(J.H. Jacobi)

During the previous reporting period, a mechanical design was developed for a sample holder to be used for measurement of complex permittivity of biological materials. This device was then fabricated by the Division of Instrumentation. The objective of this approach was to develop a technique that was broadband (at least one octave), simple to use and did not require solution of transcendental equations (transcendental equations with complex arguments do not have a single solution).

In the current reporting period, considerable progress was made in developing the device into a usable laboratory tool. A paper was presented at the International IEEE Antenna & Propagation Symposium in June 1977, as summarized below.

A mathematical model was developed that describes the sample holder and interposed transmission line. Using this model, a theoretical technique was developed for calibrating the measurement system using a compound whose complex permittivity is known. This method allows determination of the electrical capacitance of the empty sample holder and the complex propagation constant of the transmission line from two measurements. The unique features of the calibration technique are that the dimensions and shape of the sample holder need not be known and the sample holder does not have to be disassembled for calibration. These are significant advantages that no other technique offers.

A program was developed which accepted the reflection coefficients of the empty sample holder, the holder filled with the known material (water or carbon tetrachloride) and the holder filled with the unknown material as inputs. From these inputs, the program calculated, as a function of frequency, the real and imaginary parts of the complex permittivity, conductivity, and loss tangent.

The calibration technique and measurement system were tested using liquids of known complex permittivity. These liquids include ethanol, methanol, ethylene glycol and benzene. The values of permittivity obtained from this system agreed with the published literature within  $\pm 10\%$ . The bandwidth of the system far exceeded the expectations. Satisfactory measurements were possible from 1.0 MHz to 500 MHz (nine octaves) with materials whose relative permittivity was less than 40.

The entire process of calibration and acquisition of data from unknowns was automated using the Hewlett Packard 8507A Automatic Network Analyzer. This permits the measurement of complex permittivity at rates which has never before been possible. For example, the unknown could be characterized at 40 discrete frequency steps in less than one minute.

Aside from the fact that this allows characterization of many materials in a short time, makes possible following the time course of changes in microwave properties in biological preparations.

The system is now being employed as a laboratory tool for microwave interrogation of nucleic acids, their component mononucleotides, and base pairs.

Future development will include coating the interior with an inert metal such as platinum, and provision for accurate control and measurement of the temperature of samples within the holder. Additionally, it is expected that the frequency range can be extended to at least 4000 MHz.

F. Electromagnetic Analysis of Membrane Function in Erythrocyte Suspensions Under Physiological and Induced Pathophysiological States (L.E. Larsen, J.H. Jacobi and A.K. Krey)

The project represents an extension of DART and dielectric relaxation studies into the area of cellular physiology and pharmacology. The work was performed largely at U.S. Army Electronic Material Readiness Activity, Vint Hill Farms, VA. where appropriate network analysis could be accomplished.

The study employed washed rabbit erythrocytes under a variety of physiological and drug induced pathophysiological states. The physiological states included isotonic saline suspension and MOPS-TRIS buffered isotonic saline at pH 6.5, 7.0 and 7.5. The drug induced pathophysiological states included various examples of membrane transport aberrations such as Na/K pump blockade with a cardiac glycoside and ionophoretic agents to alter membrane conductivity to anions.

The other pathophysiological states were hemolysis induced by normothermic sonication and minor osmotic shifts produced by 10  $\mu$ L doses of water in 1 ml blood sample.

Briefly, the experiments disclosed an HF band relaxation frequency in the cell suspensions which we attribute to the cell membrane since this dispersion disappeared in sonicated samples. In addition, transport blockade with ouabain was detected by declining intracellular dielectric conductivity due to increased intracellular water concentration and/or cell swelling. Alterations in the same direction with a 10  $\mu$ L  $H_2O$  dose were also observed. This effect had smaller magnitude and different time course than that due to ouabain.

The 3 pH states were clearly separable by dielectric conductivity. As the pH became more alkaline, the lower frequency values demonstrated progressively lower dielectric conductance. We believe this represents alteration in cell surface charge due to pH dependent polyanionic exo-proteins of the cell membrane.

Ionophore studies indicated the effect of channel conductivity changes when ionophore treated cell suspensions were tested with a 30  $\mu$ L dose of 3 M KCl. The ionophore treated cells showed reduced dispersion of dielectric conductivity. We interpret this to indicate reduced membrane ability to maintain concentration gradients under the experimental condition.

These results have been submitted for publication in the scientific literature.

Future directions in this project will include extension of these observations to other tissues and pathophysiological states.

G. Microwave Interrogation of Nucleic Acids and Their Component Mononucleotides and Base Pairs (A.K. Krey, J.H. Jacobi and L.E. Larsen)

Essential for the genetic expression of DNA is the complementary base pairing scheme which has been proposed for the nucleic acid with the introduction of its double-helical secondary structure (5), but which also applies to RNAs and to DNA-RNA hybrids involved in genetic events. The scheme provides that DNA replication and RNA transcription yield newly formed products whose sequence of bases is complementary to that of the original template strand and that in protein synthesis codon triplets of messenger RNAs are recognized by anticodon-triplets of transfer RNAs which are of complementary nature. Because of the importance of complementary base recognition in key cellular biosynthesis reactions, the earlier estimates by non-microwave techniques of relaxation frequencies for the complementary base pairing which would fall right into the microwave region was reinvestigated by interrogating DNA, RNA and their constituent mononucleotides with microwaves. The results are described below.

Absorption of microwaves was determined by network analysis (Vint Hill Farms Station, VA). The raw absorption data were converted to permittivities ( $\epsilon'$ ) as function of microwave frequencies, yielding the upper-frequency portion of the relaxation region of the different compounds under investigation.

We have confirmed a DNA dispersion but have been limited -- due to the lower-frequency limit of the available network analyzer -- to the upper frequencies of the dispersion region. In addition, we have observed for one kind of RNA -- transfer RNA -- a similar relaxation. This relaxation of DNA and RNA may correspond to the relaxation of constituent mononucleotides of the nucleic acids or their bases and the complementary pairs they are known to form.

We have therefore investigated the relaxation of biologically important mononucleotides or bases for three sets of complementary DNA and RNA constituents and their respective pairs. For each of the com-

plementary pairs investigated, the purine participant shows that larger changes in permittivities compared to the pyrimidine partner while the mixture of two partners shows a non-additive dielectric behavior, indicating probably the formation of the respective base pair. A side-by-side comparison of the relaxation phenomena observed for the three investigated base pairs shows that permittivity changes are larger for the G·C than they are for the A·T and A·U pairs, reflecting either the difference in dipole moments one obtains for the compositionally differing base pairs from the moments of the individual bases (6), or a contribution to the dispersion from the phosphate part of the G·C pair (A·T and A·U were used because of the solubility reasons in the form of the bases), or both. That at least the phosphate part in nucleotides contributes to the observed permittivity changes is supported by the finding that only thymidine phosphate but not thymine or thymidine goes -- paired or unpaired with adenine -- through a dispersion region. Whether additionally base specificities enter into permittivity changes for the component mononucleotides of DNA and RNA and the base pairs, remain to be seen.

In conclusion: The present results on nucleic acids and their component nucleotides and complementary base pairs suggest that microwave interrogations of biopolymers may become a valuable tool for structural investigations of biologically important macromolecules and the function they perform when they participate in key cellular biosynthesis reactions.

#### H. Microwave-Induced Conditioned Taste Aversion Learning in the Rat (G.R. Sessions)

The purpose of this study was to make use of a sensitive aversion conditioning paradigm to determine if the effects of microwave irradiation are sufficiently aversive to support a particular type of avoidance learning. In addition, the study was designed to determine the relationship between core temperature elevation, taste aversion and incident microwave power density.

Many animals, and especially rats, appear to be particularly prepared to learn to avoid the taste of foods that have made them sick. This type of learning has been labeled "bait shyness" or conditioned taste aversion (CTA) learning, and has been used as a sensitive method for determining thresholds for aversive aftereffects associated with a wide variety of poisons, drugs and ionizing radiations. The rationale behind the CTA method is simply that an animal will associate just about any type of illness or malaise that is experimentally induced with the taste of any novel food or fluid that was ingested immediately prior to becoming ill.

The investigation of the dose-response relationship between microwave irradiation and CTA learning involving exposing 180 - 215 g. rats to various power density levels of radiation immediately after they had consumed a normally palatable taste stimulus, a 0.1% solution of saccharin-flavored water. Groups of water-deprived rats were given five daily

pairings of saccharin drinking followed by one-half hour irradiation with a horizontally-polarized 918 MHz, CW (continuous wave) signal at power density levels of 0, 17, 33, 25, 42 or 51 mW/cm<sup>2</sup>. This frequency and polarization were selected to optimize energy coupling from the incident field into the experimental animal.

A 30-minute two-bottle preference test between water and saccharin was conducted three days following the last irradiation.

The results are expressed in terms of the mean percent preference for saccharin solution relative to total fluid intake for the sham-irradiated and irradiated groups. All irradiated groups showed a reliable decrease in preference for the saccharin solution following five pairings. However, there was no reliable correlation between power level and saccharin preference between 17 and 33 mW/cm<sup>2</sup>. The dose-response relationship was clearer at the highest power levels of 42 and 51 mW/cm<sup>2</sup>. These results may be compared with changes in colonic temperature measured before and after irradiation.

At 17 mW/cm<sup>2</sup> there was no reliable increase in colonic temperature following 30 minute irradiation, but at power levels of 25 mW/cm<sup>2</sup> and higher, colonic temperature increased monotonically between 1.5 and 3.0 degrees Celsius.

It is probable that the taste aversion conditioning observed at power levels of 25 mW/cm<sup>2</sup> and higher resulted from the association of the discomfort due to heat loading with the taste of the saccharin consumed immediately prior to irradiation. However, this interpretation cannot at present be used to explain the slight decrements in preference for saccharin observed in the 17 mW group.

The conditioned taste aversion technique appears to be a very sensitive indicator of changes in behavior following exposure to microwave irradiation. At higher power levels, temperature elevation appears to play a role. Future research will be directed at investigating dose-response relationships at power levels below 17 mW/cm<sup>2</sup> where core temperature incremental elevations are not prominent.

#### I. Neural Membrane Effects of Microwave Radiation (P.V. Brown and L.E. Larsen)

Microwave radiation of 12.5 cm wavelength at 11 mW/cm<sup>2</sup> CW for a duration of 30 min (2 °C heating) has been shown to increase conduction rates and shorten refractory periods in amphibian peripheral nerve. Radiation with peak powers of 14 to 70 mW/cm<sup>2</sup> in pulses 1 ms. and PRR of 5/sec over a duration of 10 to 60 sec. was found to alter the compound action potential. Similar results were obtained with 10 cm waves of higher power density (370 mW/cm<sup>2</sup> peak) in the form of 1  $\mu$ s pulses (PRR 700/s) where heating was limited to 0.2 °C. These and other findings suggest a fundamental interaction between excitable membranes and micro-

wave radiation which may not be produced by "equivalent" thermalization (7). Any microwave effects which are related to excitable membranes are especially relevant points of departure for a rational discussion of safety standards.

Electrophysical studies of the type described above are complicated by the fact that recording electrodes alter the induced field and often locally increase power absorption. As a result, dosimetric statements are often difficult to support. In addition, peripheral nerve studies only indirectly address the question of macromolecular mechanisms responsible for the changes in permeability which constitute the action potential.

Alternative preparations and dependent variables are available. Specifically, the giant axon is known to display changes in the optical properties of the axoplasmic membrane which are directly related to the propagation of an action potential. In this way, membrane events are directly studied without the need for instrumentation within the biological preparation. It is these measures of membrane function which will be examined for evidence of microwave effects on axoplasmic membrane conducting an action potential. The question of induced field strength may be approached by IR pyrometry since the effective spot size can be as small as 175  $\mu\text{m}$  on an axon 500  $\mu\text{m}$  in diameter. Alternatively, exposures may take place in a transmission line where indirect measures of absorbed energy are possible.

The preparation chosen for this study was the giant axon of *Myxicola Infundibulum*. These marine invertebrates are available year round, unlike squid which are only available for a short season. *Myxicola* also may be kept in sea water aquaria for months, unlike squid which show signs of degradation within two days after capture. The *Myxicola* giant axon is about 300 to 400 microns in diameter. It is the major occupant of a dorsal envelope immediately adjacent to the dorsal muscle wall. The disadvantages of this preparation are the ill-defined length of the axon, a segmented structure, and tedious dissection procedures required to completely clean the axon. In comparison with the disadvantages of other preparations (cray fish, cockroach, lobster and aplesia), we believe *Myxicola* represents the best compromise.

In the present report period, a sea water marine lab has been established, and many score of animals have been successfully maintained. A special food has been developed which prevents weight loss in animals kept for several months. Also the optical analyzer has been successfully interfaced with the irradiation system. Since it is important to prevent field aberrations, the optical train must be external to the irradiation system.

Action potentials have been recorded from the giant axon in situ and in vitro. The dissection procedures has been carried to the extent described in the literature (8). The major technical problem remaining

is the need to extend the dissection to the point of removing all adjacent tissue without damaging the axon anywhere along a 3-4 cm length. Enzymatic degradation has been tested as a means to assist the cleaning procedure. It has been verified that enzyme treatment per se does not prevent propagation of action potentials, but the conduction velocity may be decreased. This aspect will require further careful study. Indeed at this point the success of the whole venture seems to depend upon cleaning the axon sufficiently for optical studies while retaining a substantially functional axon.

The microwave side of the experiment has been built and tested. This included the successful development of a nerve chamber in which a uniform microwave field may be induced. Thermographic measurements verified uniform heating ( $\sim 0.25$  °F) in a chamber which employed a combination of field shaping and dielectric matching to achieve uniform interior fields. A technical note on this chamber has been submitted for publication in the scientific literature.

#### J. Microwave Exposure Quality Assurance (M.P. Toman)

During the fiscal year 1976-1977 along with maintaining support functions within the Department of Microwave Research, the Microwave Engineer was involved in the following projects:

1. A number of extended measurements was made in Chamber C, including:
  - a. a detailed recheck of the chamber calibration;
  - b. a field-mapping of the electromagnetic field at an exposure distance of 8' was obtained;
  - c. the effect of the field on the measurement apparatus was determined.
2. The error analysis in regard to calibrating the chambers was restudied, and some refinements were made to it to try to understand in more detail the major contributing sources of error.
3. New exposure stands were designed and constructed to be used in Chamber C.
4. New shielded enclosures were designed and constructed to be used for chamber calibrations. Equipment used during calibrations now will be shielded from "pick up", thus allowing more accurate measurements to be made.
5. Just before leaving due to reconstruction Chamber A was rechecked and found to be in calibration.

#### K. Electronic Support (M. Swinnen)

1. A digital temperature alarm was constructed. The device permits a researcher to set a minimum and a maximum allowable temperature limit by means of two sets of thumbwheel switches. Besides continuously indicating temperature in the exposure chamber on a three-digit LED indicator, the alarm buzzer and a blinking light are activated when

aperture is outside preset limits, while a relay interrupts power to the transmitter.

2. An existing automatic transmitter tuning device was perfected, so as to make its use safer to the operator.

3. In the same enclosure, an over-power alarm was built to guard against this type of failure during exposure of microwave radiation of experimental animals.

4. Numerous calculator programs for the SR-52 were developed. Among them can be cited:

- a. 6 programs dealing with all the possible configurations of the state-variable filter.
- b. 3 programs concerning the multiple-feedback filter.
- c. 1 program about odd-resistance calculation.
- d. 1 program dealing with a random number generator.
- e. 1 program about microwave rectangular waveguide calculation.
- f. 1 histogram generator program.
- g. 1 bar graph plotter program.
- h. 1 linear-exponential curve fit program.
- i. 2 base conversion program, decimal to any base and any base to decimal.
- j. 1 parallel resonance program.
- k. 1 vector addition program.
- l. 1 time constant program.
- m. 1 single layer coil calculation program.
- n. up to now 8 programs have been published in the Texas Instrument Professional Exchange software catalog.

L. High Speed Enzyme Inactivation In Situ by Microwave Energy Application (P.V. Brown)

In the present report period, the Microwave Research Department continued to support the Medical Neurosciences Department by further refinement of the modified Varian microwave source used for neurochemical analysis in rats and mice. The techniques developed in this department are the subject of a publication (in press) which is abstracted below:

"The use of microwave energy to inactivate enzymes by rapid heating permits measurement of heat stable metabolites with minimal postmortem artifact. Reproducible results, however, require that the energy applied (dose) and the exposure geometry be reproducible. The commercial systems that we have examined are limited in this regard. A method to achieve a reproducible dose by electronic control of the applied power and exposure time is described. The electronics are compatible with any 220 volt, single phase commercial microwave power source. Without electronic control, exposure time varies by 50 milliseconds, due to random contacter closure time. Output power varies by 15%, due to tube aging and variations in line voltage, magnetron temperature and load impedance during exposure. With the addition of electronic circuitry, the exposure time can be controlled within 3 milliseconds, and the power within 2%. Timing is done by counting energy bursts from the triac controlled magnetron.

Leveling is accomplished using a feedback loop to vary the magnetron magnet current to keep the output power constant. The use of these electronic controls makes microwave irradiation systems more reliable as a biochemical research tool."

In addition, the microwave support engineer has been trained in the maintenance and operation of the inactivator. Also, an RFP was prepared for the construction of a higher powered and still more closely controlled inactivator with design principles based upon our experience with the Varian unit.

#### IV. Research Activities: Extramural Program

The WRAIR microwave exposure facilities were used for extramural activities on the following bases: 1) formal letter of agreement, 2) ad hoc, and 3) contracts.

Users with which we have a formal letter of agreement are the Armed Forces Radiobiological Research Institute (AFRRI) and the Environmental Protection Agency (EPA). Their uses of the microwave exposure facilities were:

1. AFRRI
  - a. Brain enzyme alterations
  - b. Cataract production, lens chemistry alterations
2. EPA
  - a. Radiation surveys
  - b. Antenna calibration

Ad hoc users of the microwave exposure facilities were:

1. Office of Naval Research  
Dr. T. Rozzell, Physiology Branch, ONR  
Dr. D. Rioch, Institute for Behavioral Research
2. USNMRDC  
Dr. M.M. Varma, Howard University  
Dr. E.N. Albert, George Washington University
3. American University  
Mr. K. Oscar (PhD thesis work)

The Army Research Office (ARO), for which we act as primary scientific liaison, and the U.S. Army Medical Research and Development Command (USAMRDC) have the following contracts:

1. ARO
  - a. In Situ Dielectric Constant Measurement Technology  
(Georgia Inst. Tech.)  
Purpose: Design and test implantable electrodes for measurement of tissue dielectric constant and loss tangent in situ.

2. USAMRDC

- a. Target/field Coupling (University of Utah)

Purpose: Body segment resonance at microwave frequencies

- b. Numerical Analysis for Enzyme Inactivation in Waveguide

(Georgia Inst. Tech.)

Purpose: Design guidance for improved enzyme inactivator

- c. Development of Improved 2450 MHz Enzyme Inactivator

d. Design, Construct and Install High Power Microwave Transmitter.

Project 3E762771A805 MICROWAVE INJURY PREVENTION AND TREATMENT

Work Unit 041 Biological interactions with and hazards of microwave radiation

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